PROTEIN DEGRADATION IN RAT SKELETAL MUSCLE: INTRACELLULAR, NON-LYSOSOMAL ENZYME SYSTEMS AND THEIR ENDOCRINE CONTROL

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TO VAHEEDA, MISHKAH AND KASHIF

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ABSTRACTS

Two manuscripts based on the work reported in this thesis were submitted and accepted for publication, the abstracts of which follow:

SOLUBLE AND PARTICULATE FORMS OF MUSCLE ALKALINE PROTEINASE SHOW DIFFERENTIAL SENSITIVITY TO ENDOGENOUS INHIBITOR(S)

Firhaad Ismail and Wieland Gevers

(Biochemistry International, In Press)

Membrane-free washed myofibrils derived from rat skeletal muscle homogenates contained a chymostatin-sensitive protease(s) which acted on associated myofibrillar proteins, at an optimum pH of 8.5, much less rapidly at low ionic strength (insoluble myofilaments) than at high salt concentrations (solubilized proteins). When the myofibrillar fraction was added to the particle-free cytosol prepared from the muscle extracts, proteins of the cytosol were also degraded, but the activity in this case was much more pronounced at low ionic strength. This was because inhibitor(s) of the proteinase present in the cytosol fraction were only effective at high ionic strength when all the myofibrillar (and associated) proteins were in solution.

The protease was separated from the bulk of the myofibrillar proteins by gel chromatography at high ionic strength. On dialysis against a low-salt buffer, part of the enzyme was

precipitated. The putative cytosolic inhibitor(s) were again only effective on the soluble enzyme at high ionic strength.

A HIGH MOLECULAR WEIGHT CYSTEINE ENDOPEPTIDASE FROM RAT SKELETAL MUSCLE

Firhaad Ismail and Wieland Gevers

(Biochim. Biophys. Acta, In Press)

A cytosolic enzyme of high molecular weight (about 500000), which attacks native or denatured proteins (*inter alia* casein, globin and hexokinase) was purified about 1000-fold from mixed rat skeletal muscles, including muscles freed of mast cells by prior treatment of the animals with the degranulator, compound 48/80. Peptides of varying size were generated from radio-actively labelled globin, but no free amino acids were formed; free tyrosine was also not released from azocasein. The pH optimum was 7.5 and the presence of an essential cysteine group was suggested because dithiothreitol (1 mM) stimulated the activity and N-ethylmaleimide (5 mM) and p-chloromercuriphenylsulphonic acid (1 mM) were inhibitors. The activity was markedly inhibited by Zn^{2+} but not by leupeptin, chymostatin or pepstatin. The enzyme was stabilized by ATP, at concentrations as low as 0.1 mM, against inactivation at 42°C. The endopeptidase was clearly separated on gel chromatography from another large protease, also sensitive to Zn^{2+}, but with marked aminopeptidase activity and the properties of Hydrolase H.

The activity levels of the protease, assayed after chromatography on Sepharose 6B of high-speed supernatant fractions, did not vary significantly in skeletal muscle samples which were derived from denervated, starved, diabetic or hyperthyroid animals, in all of which the abnormal physiological states expressed themselves as enhanced rates of tyrosine release by incubated soleus and extensor digitorum longus muscles. Nevertheless, the enzyme described here may be part of an ATP-dependent, multi-component proteolytic system similar to that already known to be present in reticulocytes.

LIST OF ABBREVIATIONS

AMP, ADP and ATP	=	the 5' mono-, di- and triphosphates of adenosine
Bz-Arg-N-Nap	=	α-N-benzoyl-L-arginine-2-naphthylamide
Ci	=	curie (3.7×10^{10} disintegrations per second)
DFP	=	diisopropylphosphofluoridate
dpm	=	disintegrations per minute
DTT	=	dithiothreitol
EDL	=	extensor digitorum longus
EDTA	=	ethylenediamine tetracetic acid
EGTA	=	ethyleneglycol-bis-(β-amino ethyl ether)
g	=	acceleration due to gravity
gm	=	gram
h	=	hour
HMM	=	heavy meromyosin
HSSN	=	high speed supernatant
i	=	ionic strength
Leu-N-Nap	=	L-leucine-2-naphthylamide
LMM	=	light meromyosin
M	=	molar concentration
MF	=	myofibril(s)
mg	=	milligram
min	=	minute
ml	=	millilitre
mm	=	millimeter
NEM	=	N-ethylmaleimide
PAGE	=	polyacrylamide gel electrophoresis
pH	=	negative logarithm of the hydrogen ion concentration

PMSF	=	phenylmethyl sulphonyl fluoride
SDS	=	sodium dodecyl sulphate
TCA	=	trichloroacetic acid
Tris	=	tris(hydroxymethyl)aminomethane
µg	=	microgram
µl	=	microlitre
w/v	=	weight per volume
>	=	greater than
<	=	less than

DECLARATION

The research work presented in this Thesis was performed in its entirety by myself, with the exception of some relatively minor aspects of the work such as histologic procedures and tissue culture (both acknowledged in the text).

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CHAPTER 1

INTRODUCTION

1.1. INTRODUCTION

The term "protein degradation" describes one or more catabolic processes by which native proteins are converted in the organism to constituent amino acids.

It would perhaps be fair to state that our current understanding of this important area of cell metabolism is roughly at the stage which preceded the discovery of ribosomes in the pathway of protein biosynthesis.

Some of the major aims of those working towards an understanding of intracellular protein degradation are (a) to provide information on the importance of this process to the overall viability of cells or organisms; (b) to identify the nature and location of individual reactions and the rate-limiting steps in the degradative sequence; (c) to resolve differences between the catabolism of intracellular proteins and those extracellular proteins that are taken into the cell; (d) to discover the means by which the degradation of a single protein can be altered, since different proteins have differing rates of degradation; (e) to identify the component(s) and mechanisms underlying the energy requirement of the degradation process, and (f) to examine the biochemical, endocrinological and pharmacological controls of the process with the eventual aim to manipulate these controls and thus restrict disease states and other disadvantageous conditions.

A review therefore, of muscle protein degradation and, more

specifically, of recent data obtained from *in vitro* animal studies, is provided in this Chapter, outlining some of the major advances in this field and finally defining the specific objectives of the author, in the context of the above generalizations.

1.2. PATHWAY(S) OF INTRACELLULAR PROTEIN DEGRADATION

1.2.1. Endocytosis, pinocytosis and membrane recycling

Lysosomes are generally considered to be the major site of intracellular digestion of externally derived proteins. Such proteins are "captured" by cells through endocytosis or by pinocytosis, processes in which bound proteins or entire droplets of extracellular fluid, respectively, are internalized in vesicles derived from invagination of the plasma membrane (Dean, 1975). Fusion of these vesicles with primary or secondary lysosomes results in the exposure of the "captured" proteins to the lysosomal proteases and thus to their complete intralysosomal digestion to the level of amino acids (Ashford and Porter, 1962). Both these processes involve the internalization of membrane material (Dean, 1978). Since the usual fate of endocytic vesicles and their contents is fusion with the lysosomes, it was assumed for many years that the incoming membrane was digested together with its contents. It is now evident that in some mammalian cells at least, much of the membrane escapes degradation and is returned to and inserted into the plasma membrane (Tulkens *et al*, 1978): Fibroblasts were allowed

to endocytose and accumulate goat (antirabbit) IgG, following which surface antigens were tagged with rabbit antiplasma membrane IgG. Trapping of antiplasma membrane IgG was observed in lysosomes as well as at the cell surface - consistent with the internalization of surface antigens by endocytosis, followed by their recycling to the cell surface. The mechanism whereby membrane is removed from and subsequently reinserted into the plasma membrane is not yet understood. A process of budding, whereby a small vesicle is formed and discharged from the parent vacuole, seems the likely possibility (Dean, 1978). This kind of reinsertion into the plasma membrane clearly requires an interaction between the cytoplasmic face of the surface membranes and the returning membrane components. A frequently observed pattern is a sequence of fusion events, first between small pinosomes and subsequently between lysosomes and the larger pinocytic vacuole. It has been suggested (Lloyd and Williams, 1978) that these sequential vesicle fusions may provide the occasion for the budding process to take place with minimal energy requirements. When any two intracellular vacuoles fuse together, the resulting vacuole will comprise, at the moment of fusion, the combined membrane and volume of the parent vacuoles. In the new vacuole thus formed there will be more membrane than is required to surround the combined volumes of the two component vacuoles. Resumption of turgidity must involve either the influx of water or the loss of membrane. Uptake of water would aggravate water balance in a pinocytosing or endocytosing

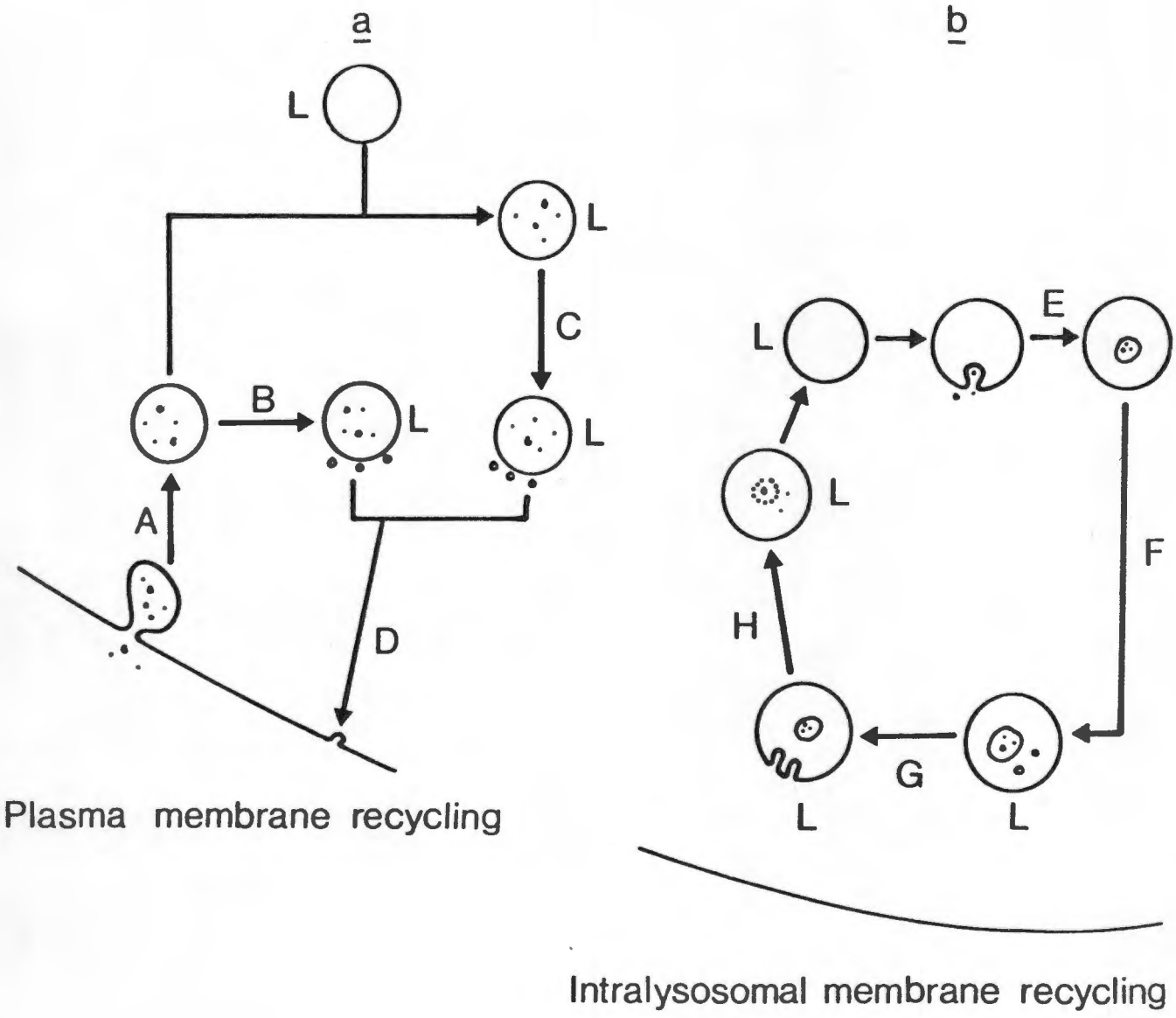
cell and hence the alternative of membrane loss appears more attractive. It would not only avoid the problem of water balance but furthermore the energy requirement for the membrane movements necessary for budding would be less if the parent vacuole had excess membrane than if it was turgid (Lloyd, 1980). Budding from pinocytic vacuoles or secondary lysosomes into the cytoplasm followed by an exocytic reinsertion into plasma membrane could represent a pathway for the recycling of plasma membrane components without degradation and resynthesis of membrane components (Fig. 1.1(a)) (Dean, 1978).

1.2.2. Lysosomes and autophagy

Internalization of non-lysosomal material into lysosomes is believed to occur either by "classical autophagy" (Dean and Barrett, 1976) or by "microautophagy" (Pfeifer, 1981).

"Classical autophagy": An area of cytoplasm newly surrounded with membrane, is considered to fuse with a primary or secondary lysosome, which are smaller vesicles containing hydrolytic activities. Digestion then occurs in the autophagic vacuole, with eventual reduction in organelle size until a secondary lysosome is formed. This lysosome is generally indistinguishable from a primary lysosome, and is able to fuse again and repeat the cycle (Fig. 1.2). The digestion of intracellular proteins by this system is referred to as "autophagy", in contrast with the uptake and digestion of extracellular material (see previous section) which is

Fig. 1.1



A - Endocytosis

B - Division to form one large and several small vesicles, alternatively a similar division may occur after the endocytic vesicle has fused (C) with a lysosome (L)

D - Small vesicles fuse with plasma membrane, thus recycling membrane

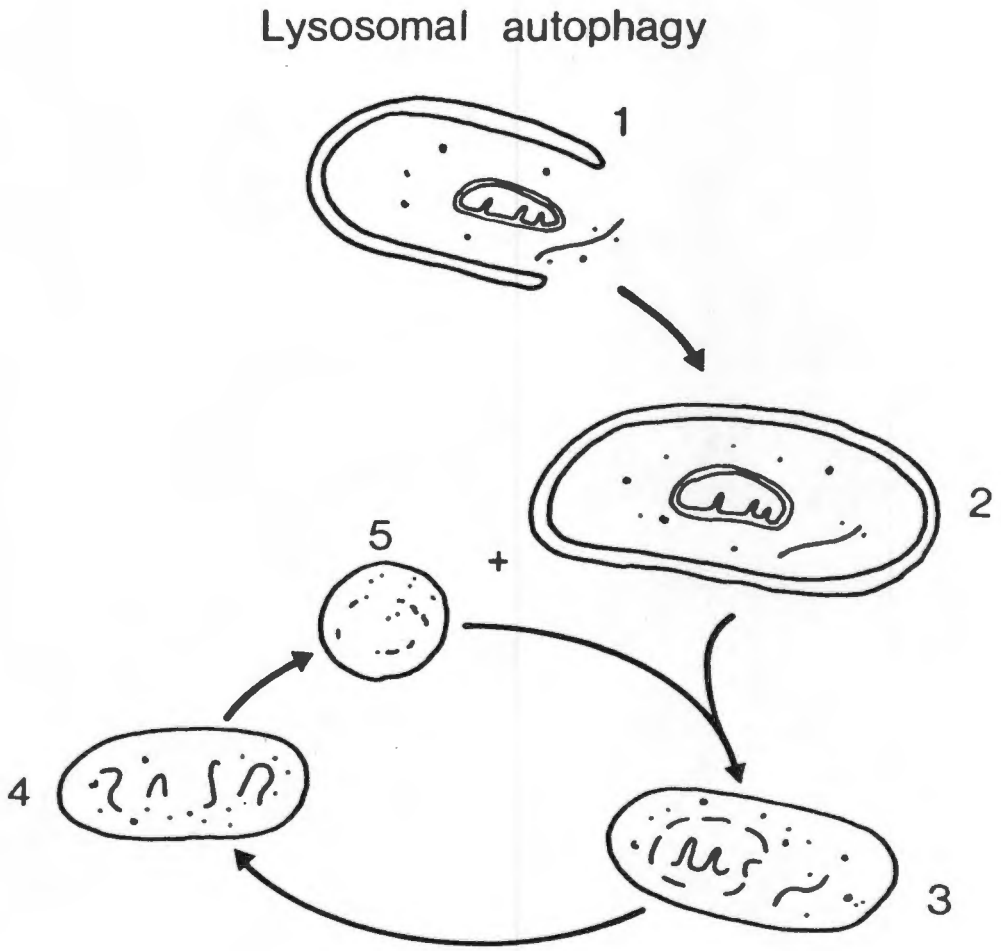
E - Lysosome invaginates to take in cytoplasmic material

F - Division of the vesicle

G - Fusion of small vesicles with lysosomal membrane (recycling)

H - Disintegration of lysosomal membrane and both lysosomal membrane and cytoplasmic protein undergo digestion by lysosomal enzymes

Fig. 1.2



A double membrane is shown enclosing a portion of cytoplasm (1) to form a vacuole (2). This fuses with a primary or secondary lysosome (5) to produce an autophagic vacuole (3). Digestion of intravascular contents continues (4) until complete, with the breakdown products passing out of the organelle, which is then reduced in size and becomes a secondary lysosome.

called "heterophagy". It is notable that endocytosed macrophage proteins are degraded in a population of macrophage lysosomes which are in at least some ways distinct from those degrading endogenous proteins, since lysosomotropic agents (likd chloroquine) inhibit the degradation of the endocytosed proteins far more than that of the endogenous proteins (Poole et al, 1977). By means of quantitative electron microscopy, a number of results have been gathered, which indicate that changes in the volume fraction of autophagic vacuoles most often correspond to changes of protein degradation as determined by biochemical methods. Such results have been most often discussed in the light of the stimulation of cellular autophagy and protein degradation by glucagon (Woodside et al, 1974; Arstila et al, 1974) and in the case of amino acid deprivation (Woodside et al, 1974). Inhibition of autophagy and of protein degradation has been noted after insulin administration (Mortimore and Mondon, 1970), during diurnal variation in food intake (Vidrich et al, 1977) and in the recovery period after protein depletion or starvation (Conde and Scornik, 1976).

Microautophagy: This is an alternative mechanism proposed for lysosomal degradation of non-lysosomal proteins. The lysosomal membrane is believed to invaginate and surround a very small portion of cytoplasm followed by internalization of this material in a vacuole (Fig. 1.1(b)). A scheme has been put forward (Dean, 1978), suggesting that recyling of

lysosomal membrane components occurs in a manner analogous to that described for endocytosis of extracellular proteins and plasma membranes (Fig. 1.1b).

By themselves, these methods of mass engulfment into lysosomes cannot account for the variety of individual half-lives obtained for different proteins in cells (Kay 1978). Under steady state conditions, the rate of degradation of each cytosolic component should be the same and should be solely a function of the rate of internalization. To overcome this problem, it has been postulated that, for each individual protein, the rate-limiting step occurs before the internalization (uptake) stage, or that there is some selectivity inherent in the uptake step itself, or that each protein has a different susceptibility to the initial proteolytic attack once inside the lysosome. For the last possibility to be rate-limiting, the proteolysis step would have to be slower than the rate of uptake, so that undegraded proteins would accumulate inside the lysosomes. If the internalized proteins had different degrees of resistance to the acid conditions and the high protease concentrations in the lysosomes, then the long-lived proteins would be those that were capable of surviving these conditions and possibly even of escaping to their native and functionally active state in the cytoplasm. Short-lived proteins would be those that were more easily denatured and proteolysed and so would survive a smaller number of passages through the lysosomal compartment. Little evidence in support of such a mechanism

has yet been obtained.

The restricted localization and predominantly acidic pH optima of lysosomal cathepsins, together with the relative instability of most intracellular proteins at lowered pH values, as well as the conceptual difficulties of allowing whole proteins to escape from the lysosomes, are considerations which suggest that, once exposed to the lysosomal conditions, virtually all proteins would be rapidly degraded, without differentiation. Thus it would seem more probable that the site of selectivity that determines the rate-limiting step for most proteins has to lie outside the lysosomal compartment.

Lysosomal proteases. It has been known for many years that most tissues contain "pepsin-like" proteolytic activity not dependent on thiol activation. The term "cathepsin" was coined for the enzyme responsible, and the first detailed study of "cathepsin" was made by Anson (1940). In 1960, Press *et al* isolated and characterized cathepsin D from bovine spleen. Since then numerous lysosomal proteases have been identified in various tissues and detailed studies of their properties have been reported.

Cathepsin D is a stable enzyme synthesized as a single polypeptide chain of about 42 000 molecular weight (Knight and Barrett, 1976). The pH optimum of cathepsin D is about 3,5 and in addition to haemoglobin, native protein substrates susceptible to the protease include cartilage proteoglycan

(Roughley and Barrett, 1977), myosin (Schwartz and Bird, 1977) and angiotensinogen (Morris and Reid, 1978). More recently, Turk and co-workers (1981) have demonstrated the existence of cathepsin D precursor, cathepsinogen D, which is converted by intramolecular activation to the active enzyme and an activation segment-peptide(s) in a way similar to pepsinogen activation.

Cathepsin B, a cysteine protease, has been isolated from and estimated in many mammalian species and tissues (Barrett, 1977)[a+b]. Its molecular weight range between 24 000 and 28 000 and it is most active at degrading synthetic substrates and native substrates at pH 5,5 - 6.

Cathepsin H, initially purified from rat liver (Kirschke et al, 1972) and maximally active at pH 6 - 7, differs from other lysosomal cysteine proteinases in that it has aminopeptidase as well as endopeptidase activities.

Cathepsin L, yet another lysosomal cysteine protease, purified from rat liver, is characterized by its particularly high activity in degrading short-lived cytosol proteins at pH 6 (Kirschke et al, 1980). At pH 3, however, the capacity of short-lived cytosol proteins to serve as better substrates than long lived proteins was not evident (possibly as a result of changes in conformation). The specificity of cathepsin L at neutral pH values is interesting. Okitani et al (1980) have shown that at pH 7 and 7,5, cathepsin L seems to act on proteins by cleaving only restricted specific peptide bonds; an example is the removal of the

Ca^{2+}-sensitivity and ATPase activity of myofibrils; at lower pH values, an overall degradation of all muscle proteins occurred.

Cathepsin N (Evans and Etherington, 1978), Cathepsin S (Turk et al, 1978) and Cathepsin T (Gohda and Pitot, 1981) have also been reported to occur in various rat tissue extracts.

Among the lysosomal proteases, cathepsin B, H and L have proteolytic activity at pH values higher than those usually found to be optimal for cathepsin D, for example (pH 3-4). The major contributor to this activity at pH 6 is cathepsin L (Kirschke et al, 1977). Cathepsin L is also the first intracellular protease reported in skeletal muscle (since chymase is now regarded to be mast-cell derived - see later) that degrades actin (Matsukura et al, 1981). Hence, the pH range of activity of the lysosomal proteases from acid (pH 3,0) to neutral (pH 7) conditions, with varying substrate specificities, lends itself to the postulate that intracellular protein degradation could well be accomplished solely by lysosomal enzymes, whether intracellularly or in some other compartment such as the endoplasmic reticulum. Neutral pH acting proteases, such as cathepsin L, could also act on the cytoplasmic face of the lysosomal membrane, carrying out limited proteolysis of susceptible hydrophobic protein substrates. These may then be internalized by micropinocytosis and the final hydrolytic events may be carried out by other proteases within the lysosomal matrix.

Pepstatin is a specific inhibitor of aspartic proteinases and as cathepsin D is the major cellular aspartic proteinase, this agent provides an effective means of specifically inhibiting this lysosomal enzyme (Barrett, 1980). On the other hand, the peptide aldehydes (leupeptin, chymostatin, elastinal, antipain - Umezawa and Aoyagi, 1977) inhibit cysteine as well as serine proteinases, although the susceptibilities of the various cysteine proteases to these different inhibitors vary with the individual inhibitor tested (Kirschke et al, 1980).

Despite the identification of numerous lysosomal proteases with acidic and neutral pH optima, together with the specific and non-specific hydrolyzing capacity of cathepsin L at differing pH values, a model for the sole participation of the lysosomal system in the degradation of all cellular proteins is unlikely to be correct. The varying specificities of the lysosomal proteases do lend themselves to the notion that some limited selectivity in the lysosomal compartment may be operative, although extralysosomal events such as altered substrate susceptibility, limited proteolysis, energy-dependent cytosolic proteolytic mechanisms and membrane attachment (uptake) are equally likely to participate at various stages in the overall selective, degradative process.

1.2.3. Non-lysosomal intracellular proteases

1.2.3.1. Protease activities in skeletal muscle

Considerable attention has been focussed on proteases with pH optima in the neutral and alkaline range, particularly as some of these enzymes have appeared to be associated with muscle-specific proteins which they can degrade. These proteases are found in both the particulate and soluble fractions of the cell. Calcium-activated protease (Busch et al, 1972) removes Z-lines from myofibrils, hydrolyses denatured casein, [^{3}H]acetyl haemoglobin and N-ethyl[^{3}H] maleimide-labelled α-actinin (Reddy et al, 1975) and is eluted from DEAE-cellulose columns by 0,25 M NaCl (Waxman and Krebs, 1978). This enzyme is widely distributed in other tissues, and in kidney and lung the activity is 2,5 and 9 times greater than in muscle (Waxman, 1979). The ubiquity of its distribution suggest functions other than muscle cell protein turnover. A similar calcium-activated or dependent-neutral protease has been described in rat skeletal muscle (Kohn, 1969), but this protease does not hydrolyze haemoglobin and may be a separate enzyme. More recently, a calcium-dependent protease has been identified which requires calcium in the μmolar range for activation, a property compatible with a physiological role for the protease in protein degradation (Dayton et al, 1981).

The form of the protease activated by micromolar Ca^{2+} concentrations appears to be less negatively charged than the form requiring millimolar Ca^{2+} as the former elutes from a DEAE-

cellulose column at a lower KCl concentration, and also has a lower mobility in non-denaturing polyacrylamide gel electrophoresis, than the latter form (Dayton et al, 1981). It is thus possible that the one form of the protease may be converted to the other by a reversible process such as phosphorylation or an irreversible process such as proteolysis. This represents an attractive model for the flexible control of activity of this protease, since the interconversion of the enzyme would produce activity or inactivity at the low free calcium concentrations normally found inside muscle cells. The isolation of an endogenous inhibitor of the calcium-activated protease (Okitani et al, 1976; Waxman and Krebs, 1978) provides a possible further component in the control of the activity of the calcium-activated protease, although further studies of the mechanism of interconversion and precise localization of the protease(s) and inhibitors in muscle cells are needed to understand their roles in intracellular muscle cell proteolysis.

Alkaline proteases that sediment in the particulate fractions of muscle homogenates include the so-called "myofibrillar" protease from rat skeletal muscle (Holmes et al, 1971; Mayer et al, 1974; Dahlman and Reinauer, 1978), and serine proteases that have been found in various preparations of rat skeletal muscle (Katunuma et al, 1975; Sanada et al, 1979) and rat heart (Murakami and Uchida, 1978). Recent evidence (aimed primarily at ridding tissues of mast cells by the mast cell degranulator, compound 48/80) have indicated that

most or all of this activity resides in mast cells rather than in muscle cells (Park *et al*, 1973; McKee *et al*, 1979; Edmunds and Pennington, 1981). In addition, measurement of this activity in isolated heart muscle cells (McKee *et al*, 1979) and more recently in striated muscle cells in culture (Bird *et al*, 1981) has confirmed that this activity resides in mast cells in un-homogenized tissues. Despite this strong evidence for non-muscle cell location of the "myofibrillar" protease, earlier reports had indicated that this activity correlated with manoeuvres aimed at enhancing muscle proteolysis (Mayer *et al*, 1974; Katanuma *et al*, 1976) suggesting that the mast cell chymase was either a limiting factor in the degradative process of muscle cell proteins (this was the view maintained by these authors) or that the enhanced chymase activity was unrelated and independent of the biochemical processes determining enhanced *in situ* proteolysis in these states. Measurements of *in situ* proteolysis of intact isolated skeletal muscles from normal and mast cell-degranulated rats, have been reported by Libby and Goldberg (1980). Their results suggest that basal proteolysis is not limited by chymase activity. McKee *et al* (1979) have observed similar results in *in vitro* experiments with the perfused heart and isolated heart cells. A comparison between altered *in situ* rates of proteolysis in skeletal muscles and assayable chymase activities is not yet available.

More recently, high molecular weight proteases have been described in liver homogenates (Rose *et al*, 1979; De Martino

and Goldberg, 1979), rabbit skeletal muscles (Okitani et al, 1981) and in human and rat skeletal muscles (Hardy et al, 1981). Although there are several properties in common between these macroproteases, the interesting question is whether such activities are related to the long-established energy requirement of the degradative system. Most interesting and novel observations in relation to the energy dependence of protein degradation in reticulocytes have been reported by Hershko and co-workers (1979, 1980, 1981) where for the first time mechanistic evidence for an ATP-dependent multi-component pathway for the degradation of soluble protein substrates has been presented. The role of ubiquitin in such a system has been clearly demonstrated (see below) and curiously a macroprotease (which by itself is stabilized in the presence of ATP against thermal inactivation) has also been identified. Only one of the above-mentioned macroproteases bears resemblance to this high molecular weight protease in reticulocytes with regard to the property of ATP-dependent thermal stabilization, namely the liver macroprotease of Rose et al (1979).

Etlinger et al (1981) have confirmed some of the earlier observations of Hershko and colleagues of an ATP-dependent proteolytic system in reticulocytes and they have further shown that this ATP-dependent system decreases with reticulocyte maturation while an ATP-independent system increased. Poly-L-lysine was found specifically to inhibit the energy-requiring system. In addition, crude extracts prepared from rabbit back and leg muscles were noted to contain an

ATP-dependent and an ATP-independent protease system where only the former was inhibited by haemin and chromatography of this material on DEAE-cellulose revealed ATP-dependent co-operativity between the adsorbed and non-adsorbed material. These investigators were unable to confirm ATP-dependent appearance of substrate-ubiquitin conjugates, and furthermore did not comment on studies comparing this ATP-dependent system in muscle extracts from exsanguinated and non-exsanguinated experimental animals.

1.2.3.2. ATP-dependent ubiquitin-dependent proteolysis in reticulocytes

One important and until recently unexplained feature of protein breakdown is that inhibitors of energy production reversibly block this process (Goldberg and St John, 1976; Olden and Goldberg, 1978). A multi-enzyme, energy-dependent proteolytic system in reticulocyte extracts, in which ubiquitin enters into covalent linkage with endogenous and exogenous substrates, has been extensively investigated by Hershko and co-workers (1979, 1980, 1981).

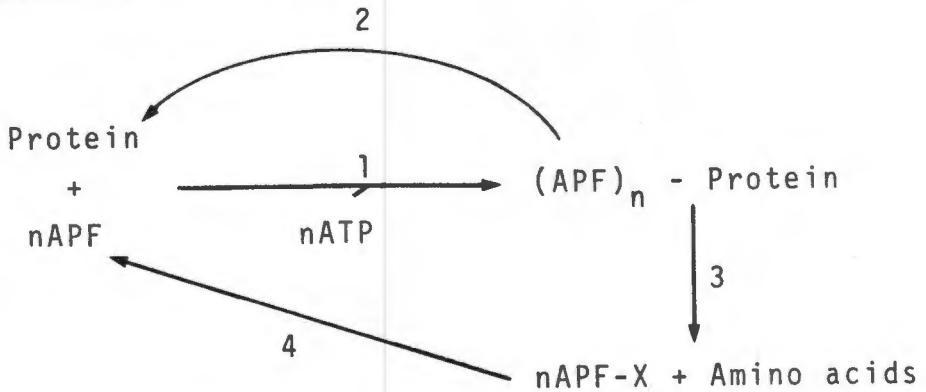
Earlier observations showed that reticulocyte lysates, prepared from ATP-depleted rabbit reticulocytes, contained little ATP-independent protease activity against denatured globin substrate, while ATP addition resulted in a marked enhancement of globin degradation by crude lysate preparations (Ciechanover *et al*, 1978). At the time, the system was resolved into two fractions in order of their elution from

DEAE-cellulose; fraction I, a 9000 dalton heat-stable polypeptide which did not adhere to the DEAE-cellulose, and fraction II, the adsorbed protein fraction - neither of these fractions by themselves showed the degree of ATP enhancement observed, while reconstitution of these fractions restored the original ATP-stimulated effect. Fraction II was subsequently resolved into two further components by ammonium sulphate fractionation, fraction IIA (0-38% cut) a heat-labile (42°C), high molecular weight protease requiring ATP for stabilization, and fraction IIB (42-75% cut), relatively stable at 42°C (Hershko et al, 1979). It was also shown by ATP trapping (with hexokinase and glucose) that all three components (i.e. fractions I, IIA and IIB) required the continued presence of ATP, and no single component was an ATP-independent terminal protease or peptidase.

Following the purification and radio-labelling the heat-stable fraction 1 (also called ATP-dependent proteolysis factor 1 or APF-1), it was shown that this polypeptide entered into high molecular weight covalent conjugates with endogenous substrates in fraction II (Wilkinson et al, 1980), and later this observation was extended to similar conjugates being formed between APF-1 and other substrates (lysozyme, globin or α lactalbumin) for the ATP-dependent proteolytic system (Hershko et al, 1980). By use of gel chromatography and $NaDodSO_4$ electrophoresis, at least two enzymatic activities were demonstrated in relation to the conjugate complexes formed, the first responsible for the synthesis of a chemical bond between APF-1 and the ε-NH_2 group of lysine

in polypeptides (termed "APF-1-protein amide synthetase"), and a second enzyme ("amidase") leading to the regeneration of APF_1 from APF-1-protein and this regenerated APF-1 was subsequently demonstrated to be re-cycled in the formation of further high molecular weight conjugates (Hershko et al, 1980).

Proposed sequence of events:



1. APF - protein amide synthetase (acting on ε-NH_2 groups)
2. Amidase that allows correction when n = 1 or 2
3. Peptidases that act strongly on $(APF\text{-}1)_n$ derivatives, when n > 1 or 2
4. Amidase for APF-1-X; X is lysine or a small peptide.

Subsequently, APF-1 has been fully characterized (Ciechanover et al, 1980[a]) and found to be a heat-stable polypeptide found universally in mammalian cells, viz. ubiquitin (Wilkinson et al, 1980). Ciechanover and co-workers have also shown that an SH-enzyme in fraction II carries out the activation by ATP of APF-1 by pyrophosphate displacement (Ciechanover et al, 1981). The adenylate is subsequently transferred to an acceptor sulphydryl on the enzyme - the nature of such a thiol ester bond being confirmed by cleavage of this linkage

by hydroxylamine, sodium borohydride as well as salts of mercury. Furthermore, this work has been extended to show that the active form of ubiquitin has the COOH-terminal sequence of Arg-Gly-Gly at residue numbers 74-76, whereas the inactive form terminates in Arg^{74}, the latter being a limited tryptic digestion product of the former during the purification procedure. Most recently, Haas and Rose (1981) have demonstrated that haemin effectively inhibited overall ATP-dependent ubiquitin-dependent proteolysis as well as conjugate degradation to exactly the same extent. A model was thus proposed in which haemin acted as a negative allosteric effector in the initial step of a sequential degradative pathway by which intact ubiquitin conjugates are first cleaved to ubiquitin-associated fragments.

Ciechanover and co-workers (1982) have purified the ubiquitin-activating enzyme by "covalent affinity" binding to ubiquitin-Sepharose. The purified enzyme has an apparent molecular weight of 210000 and appears to be composed of two subunits of 105000. Subsequent to this, Haas et al (1982) have shown that the ubiquitin-activating enzyme catalyzes the reaction:

$$2ATP + 2Ub + ESH \xrightarrow{Mg^{++}} 2PP_i + AMP + E^{AMP\text{-}Ub}_{s\text{-}Ub}$$

with the formation of enzyme-bound COOH-terminal ubiquitin thiol ester and a COOH-terminal ubiquitin adenylate. These observations indicate that ATP hydrolysis is coupled to the formation of activated ubiquitin intermediates that have

the energy to proceed to conjugate formation in the absence of additional ATP. Further investigations into the general role of ubiquitin have been reported by Matsui et al (1982) where an enzyme termed isopeptidase has been identified, in eukaryotic cells, which cleaves A24 stoichiometrically into histone H2A and ubiquitin. They propose that the attachment of ubiquitin to core histones (H2A and H2B) leads to the repulsion of adjacent chromatin fibres and that this relaxation of chromatin fibres, in turn, enables non-histone chromatin protein such as regulatory proteins and RNA polymerases to bind to accessible genes.

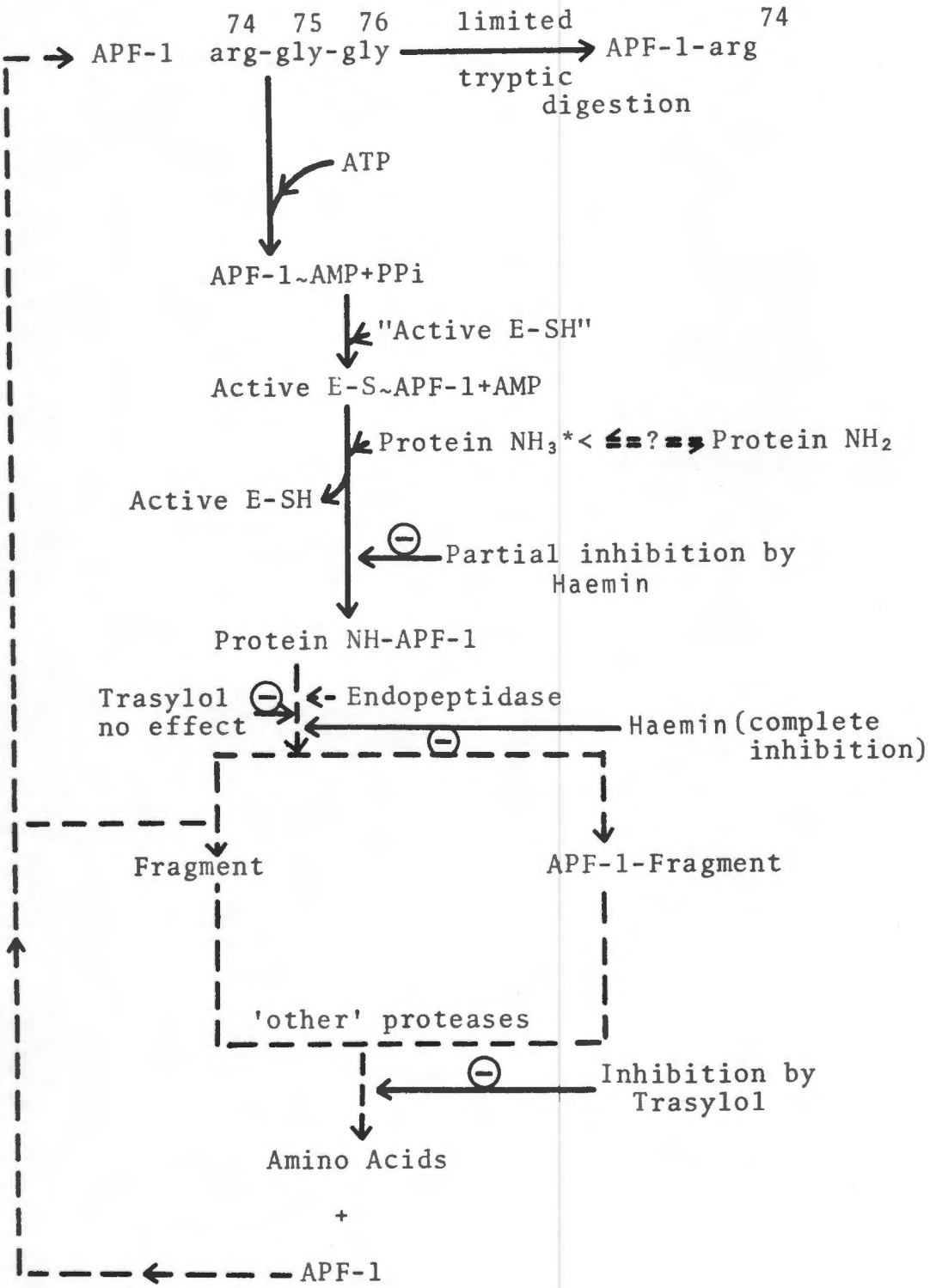
Research, therefore, in elucidation of the molecular details of the energy requirement of proteolysis has led to the identification of several components in this process in which the participation and dependence upon ubiquitin has clearly been demonstrated. A schematic model, based on results obtained by Hershko and co-workers for reticulocytes, is presented (Fig. 1.3). While novel observations have been made, further studies with selective inhibitors of the selective steps are necessary to elucidate the precise sequential stages in this proposed pathway, to discover whether it has general significance in the cells of the body other than reticulocytes, and to define its overall intracellular roles.

1.2.4. Endogenous inhibitors of proteases

With growing interest in intracellular proteinases in recent years, there has also been concomitant interest in the possible

Fig. 1.3

ATP-DEPENDENT UBIQUITIN-DEPENDENT PROTEOLYSIS:



APF-1 = ATP dependent proteolysis factor or active ubiquitin arggly-gly

"Active E-SH" = Activating thiol enzyme

(---------→) = proposed though not proven reaction steps

⊖ = inhibitor effect

presence of intracellular inhibitory substances. Such inhibitory substrances have the potential for masking the activity or presence of proteinases in crude muscle homogenates, and may also be important regulatory agents in protein degradation.

Noguchi et al (1972, 1974) reported that an inhibitor was present in the supernatants of muscle homogenates. This factor inhibited autolysis of muscle tissue in the alkaline pH range. The inhibitor had properties similar to those of serum α_1-trypsin inhibitor. Katunuma and colleagues (1977) reported a specific inhibitor to their "group-specific protease", isolated from skeletal muscle tissue. This inhibitor appeared to be a peptide with an apparent molecular weight of less than 5000 daltons, which existed in the same subcellular fraction as the proteinase. An inhibitor (mol. weight 8000) in muscle supernatant fractions was isolated by Beynon and Kay (1978); the activity of the inhibitor decreased when animals were fed protein-free diets or were starved. Afting et al (1979) have reported a proteinase inhibitor in rat uterine myometrium which has the characteristics of a glycoprotein and an apparent molecular weight of 90000. It inhibited a neutral proteinase from uterus that co-purified with the actomyosin complex and which degraded myosin. The amount of inhibitor increased 15-fold during pregnancy and then decreased to normal levels during post-partum involution. The authors suggested that the inhibitor regulated the action of the neutral proteinase on actomyosin,

since actomyosin is rapidly degraded only after parturition.

Waxman and Krebs (1978) have reported the isolation of two proteinase inhibitors from extracts of bovine cardiac muscle homogenates; a 270000 dalton inhibitor showed specific activity towards the calcium-activated proteinase from this tissue, while the second inhibitor (70000 dalton) was a trypsin inhibitor. In addition, reports of inhibitors of the lysosomal cathepsin B and H have appeared (Lenney et al, 1979), and more recently an endogenous thiol protease inhibitor from rat liver has also been described (Kominami et al, 1981).

The identification of protease-specific inhibitors in muscle extracts creates the possibility that the activity levels of proteases in subcellular compartments may remain unchanged, but that in situ activities of such enzymes may be regulated by modulation of inhibitor activity. Such inhibitor effects could vary depending on the presence of active or inactive forms of the inhibitor, compartmentation and access control between inhibitor and specific protease(s), while differential enzyme susceptibility to inhibitors could be a further factor. Thus, enzymes could exist in susceptible (soluble) forms or insoluble or particle-bound forms. Blood-borne inhibitors must also be taken into account, in terms of a possible influence on intracellular degradative events. With regard to this possibility, it has already been demonstrated (Stauber et al, 1981) that some α_1 anti-trypsin can be located within muscle cells and that its levels may be correlated with

states of enhanced proteolysis (see under 'Methodological considerations').

In one system of rapid, physiological proteolysis, the involuting uterus, Afting and his colleagues have postulated control of proteolysis to be exerted mainly at the level of inhibitor content of the cells rather than by the activity of protease (Afting et al, 1979). Further insights into these types of interactions will clarify the role of endogenous inhibitors in the pathway(s) of protein degradation.

1.2.5. Pathway Models

Proteins with large subunits are in general degraded more rapidly than those with smaller molecular weight subunits. This has been shown by studies utilizing mainly the double-labelling techniques (Dice et al, 1979; Momany et al, 1976). The correlation was not so marked when expressed on the basis of multimeric molecular weights of the intact proteins. Thus it apears that intracellular multimeric proteins may disaggregate as a pre-requisite for degradation, which then takes place at the level of the subunits.

The molecular property of surface charge expressed as isoelectric point has also been correlated with half-life of groups of proteins inside cells. Thus acidic proteins are degraded more rapidly than basic ones (Dice and Goldberg, 1976). Furthermore, it has been proposed that the distribution of hydrophobic amino acid residues between the

interior and the surface of each protein could be a determinant of its degradation rate (Ballard, 1977).

Rapidly turning over proteins appear to have a greater general hydrophobicity since they accumulate preferentially at aqueous/organic solvent interfaces and bind to hydrophobic chromatography resins such as phenyl-sepharose with greater affinities than do proteins with long half-lives (Bohley and Riemann, 1977). The possibility that selective binding of proteins to lysosomal surfaces might lead to their selective internalization and degradation has been proposed before (Dice and Goldberg, 1976). Selective binding of proteins to hydrophobic surfaces (Bohley and Riemann, 1977) and to external surfaces of lysosomes and other membranes (Dean, 1975[a]) has in fact been shown to occur. For example, Bohley and associates (1981) found that the hydrophobicity of cytosol proteins from rat liver homogenates (prepared from rats fed a protein-free diet) was diminished in comparison with the affinity of cytosol proteins from normal or protein-fed rats. Such changes in the state of cytosolic substrates may play a rate-limiting role in the transfer of these proteins to other organelles (e.g. microsomal membranes or membranes of lysosomes), for their ultimate degradation to amino acids. A further observation links the half-lives of intracellular proteins *in vivo* to their susceptibility to attack by endopeptidases *in vitro* (Yamasaki and Ichihara, 1976). Cytoplasmic proteins with short half-lives (as measured from isotope ratios after double-labelling experiments) are more

susceptible to digestion at neutral pH by extracellular enzymes, such as trypsin and pronase, than their long-lived counterparts.

More recently, Holzer (1981) has shown that yeast fructose 1,6-bisphosphatase undergoes covalent conversion (metabolic interconversion) by phosphorylation of a serine-residue and this form of the enzyme may show a different susceptibility to proteinases. In addition, it has been shown (Bond and Offermann, 1981) that disulphides (oxidized glutathione or cystine) reversibly inactivated native rabbit muscle aldolase resulting in unstable protein conformations which were very extensively degraded by lysosomal and non-lysosomal proteases. This suggests that the formation of such protein-glutathione mixed disulphides may be the initial step in the degradation of proteins where the relative susceptibility of -SH groups on soluble proteins to such oxidations may explain the heterogeneity of enzyme half-lives. Pace and associates (1981), in their studies relating to the stability of globular proteins, indicate that about 30% of accessible side chains of a typical globular rpotein are nonpolar, while over 80% of the buried side chains are non-polar. Thus, dissociation, unfolding or any other conformational change will yield a considerably more non-polar molecule which, in turn, will increase the affinity of the molecule for lysosomal or other membranes, and hence could be an important determining factor in the selective degradation of cellular proteins.

The molecular properties of substrate proteins which may thus be important in determining degradative rates include molecular weights, iso-electric points, hydrophobic amino acids on the surface, phosphorylation, and other covalent modifications (e.g. formation of mixed disulphides) and the general conformational stability of globular proteins. However, substrate susceptibility is clearly only one of several steps in the degradative process which may be rate-limiting. Other stages which may equally be regulatory will be discussed below.

A variety of studies from different laboratories have indicated that mammalian cells contain more than one proteolytic pathway that may serve different functions. For example, the degradation of long-lived proteins in hepatocytes and fibroblasts differs from that of short-lived proteins in its sensitivity to decreased temperature, to inhibitors of lysosomal function (such as leupeptin, chymostatin or chloroquine) and to nutritional step-down conditions (Hopgood et al, 1973; Amenta et al, 1976, 1977; Knowles and Ballard, 1976). If a single proteolytic pathway were responsible for the degradation of all intracellular proteins their half-lives would be expected to change similarly in responseto these experimental manipulations. However, serum deprivation of cultured mammalian cells seems to promote selectively the breakdown of long-lived proteins without affecting the half-lives of short-lived cell constituents (Knowles and Ballard, 1976). Furthermore, addition of insulin or serum can depress the

degradation of long-lived proteins in cells (e.g. hepatocytes) without affecting the rapid breakdown of analogue containing or normal short-lived proteins; and under conditions of nutritional and hormonal deprivation, when the lysosomal pathway is supposed to predominate, autophagic vaculoles are very pronounced (Amenta et al, 1977). Together, these in vivo findings suggest that long-lived proteins, which constitute the bulk of hepatocyte proteins are catabolized by a lysosomal process or that some steps involve the lysosomes. In addition, Mortimore and Schworer (1980) have demonstrated a rather strong correlation between overall proteolytic rates, the aggregate volumes of lysosomal elements, and measurements of degradable intra-lysosomal protein in states of nutritional deprivation and enrichment. This is further support for the view that intracellular proteins, with the exception of some rapidly turning over components, are degraded intralysosomally. However, Dean (1980) has demonstrated that pepstatin, the inhibitor of lysosomal cathepsin D, used with liposomes as a vector, not only inhibited accelerated proteolysis in the perfused liver, but a substantial degree of 'basal' proteolysis as well. Although the lysosomal participation in protein degradation in accelerated proteolysis is well established, the case is not the same for 'basal' proteolysis, and therefore the existence of partially separate systems has to be postulated to explain these observations. In contrast, a non-lysosomal, energy-dependent proteolytic system presumably catalyses the rapid breakdown of aberrant proteins and various normal proteins with short half-lives (Goldberg et al, 1980[a]).

There is little evidence for the accumulation of partially degraded protein products *in vivo*, suggesting that once a protein molecule is launched into the breakdown system, it is rapidly degraded to amino acids (Kay, 1978).

Thus the evidence that certain agents have marked effects on the turnover of one category of proteins (the long half-life proteins) but little or none on other classes of proteins, strongly favours a dual pathway model for intracellular protein degradation. However, from the preceding discussion, the rate-limiting step(s) of selective degradation of cellular proteins may be situated at the level of substrate susceptibility (with the various mechanisms that may alter this), early selective limited proteolysis by cytosolic proteases, membrane attachment/uptake (lysosomal or other) or selective intralysosomal attack. Furthermore, the isolation of endogenous inhibitors (ref. 1.2.4) of proteases adds further complexities to the construction of a satisfactory model. The roles of organellar compartmentalization of proteases and their inhibitors, variations in the absolute levels of these inhibitors and proteases, interconversion of inhibitors/proteases into active or inactive forms and restricted specificity of inhibitors/proteases at the level of rate-limiting steps all require careful evaluation. When the activities of protease(s) and inhibitor(s) do not correlate with alterations in protein degradation rates *in situ*, no firm conclusion can be drawn about their participation in the overall degradative process, since they may not be involved at metabolic

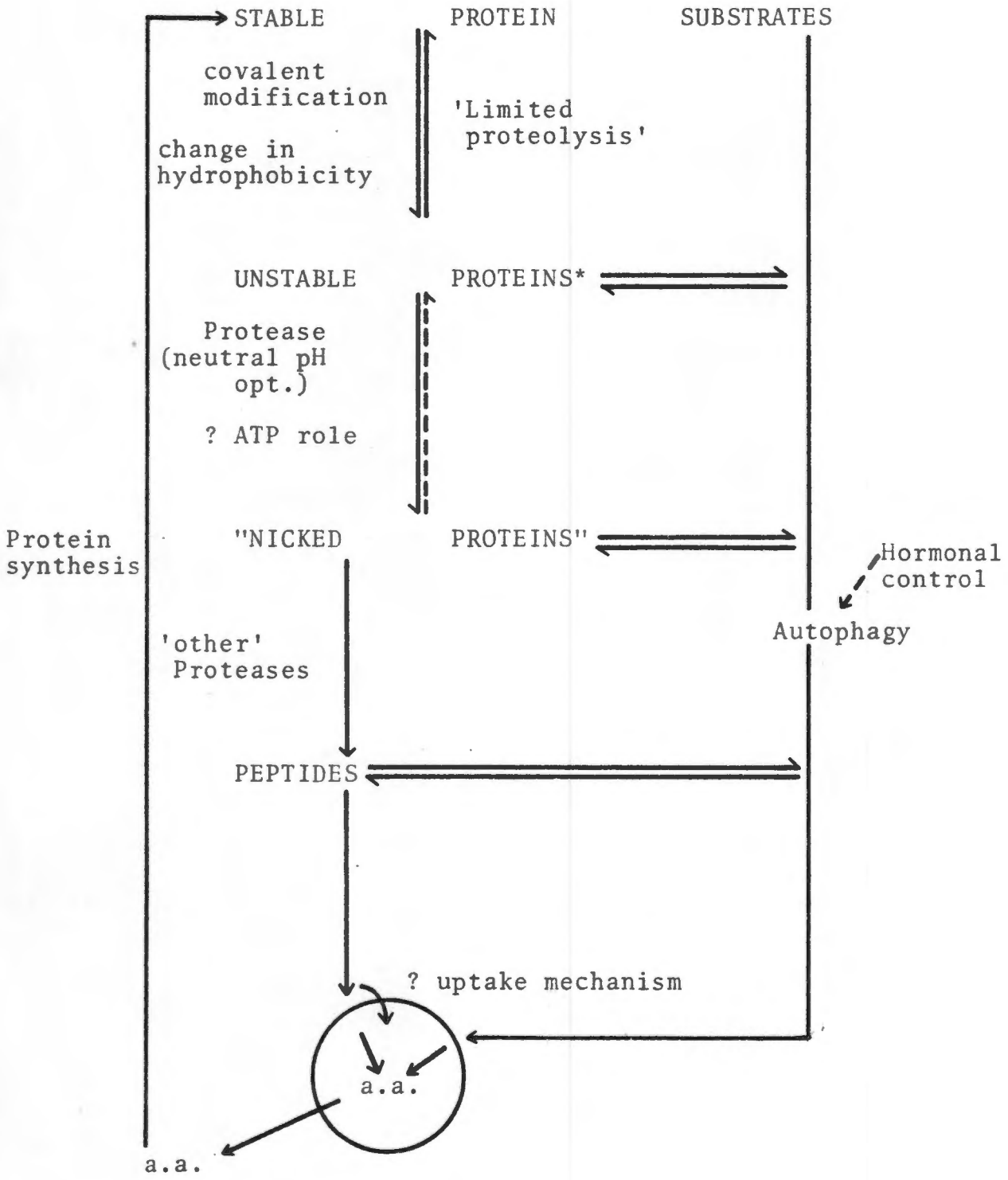
control points. However, as the identity of individual reactions, precise sites of rate-limiting steps, effects of endogenous inhibitors, determinants of substrate susceptibility and the overall control mechanism(s) remain unresolved questions, the details of most models for intracellular protein degradation at this stage are in the main speculative (Fig. 1.4.).

1.3. THE ENDOCRINE (HORMONAL) REGULATION OF PROTEIN DEGRADATION IN SKELETAL MUSCLE

The nett balance between protein synthesis and protein degradation determines whether any muscle undergoes growth or atrophy (wasting) (Goldberg and St. John, 1976). This balance varies under a variety of conditions, including accelerated growth, starvation, various endocrine disease states (e.g. hyperthyroidism, diabetes mellitus, Cushing's Syndrome) and increased use or disuse of muscle. Furthermore, as muscle protein constitutes an important fuel reserve in the body, enhanced proteolysis and the production of amino acids during this process, with the subsequent metabolism of the glycogenic precursors in the liver and kidney cortex, serve metabolic adaptation during periods of increased demand. Therefore protein synthesis and degradation in skeletal muscles is likely to be under careful regulation, and in this context hormonal control forms an important mediator of these effects in the various physiological and pathological states. Several laboratories have employed *in vitro* techniques to analyse rates of protein degradation and synthesis

Fig. 1.4

DUAL PATHWAY MODEL FOR INTRACELLAR PROTEIN DEGRADATION



(a.a. = amino acids)

under carefully controlled conditions using thin rat or mouse muscles (e.g. diaphragm, red soleus or pale extensor digitorum longus muscles) (Fulks et al, 1975; Libby and Goldberg, 1980). Rates of incorporation of [^{14}C]tyrosine or phenylalanine into the incubated muscle represent protein synthesis, after correcting for intracellular specific activity, while protein degradation rates are measured by determining the nett release of tyrosine from cell protein (for further details regarding this in vitro methodology - refer (1.4) methodologic considerations).

1.3.1. Influence of in vitro conditions on protein turnover in isolated rat muscles

The in vitro measurement of amino acid release from muscles incubated in unsupplemented Krebs-Ringer bicarbonate buffer shows that nett protein breakdown occurs under even the best conditions. The muscle thus resembles skeletal muscle during a short fast in vivo. To such a system in vitro, various factors have been added to study their influence on protein balance (Fulks et al, 1975), and most of our knowledge in the area has accrued from such experiments.

Insulin: In isolated intact muscle, insulin inhibits protein degradation and simultaneously enhances protein synthesis. The inhibition of proteolysis has also been observed in these muscles in the presence or absence of glucose, which also inhibits protein degradation. Thus, the ability of insulin to reduce muscle proteolysis must be independent of its effect

in stimulating glucose uptake by muscle. This response to insulin has subsequently been observed in isolated adult myocytes and in heart cell cultures (Frelin, 1980).

Glucose: Fulks and co-workers (1975) have demonstrated that glucose addition inhibits protein degradation, while protein synthesis is unaffected. The addition of insulin to the glucose-containing medium appears additive in improving overall protein balance in muscle, although such isolated muscles are still in a state of nett breakdown in the presence of both these factors.

Plasma amino acids retard protein breakdown and stimulate protein synthesis, although only one amino acid, leucine, by itself influences protein turnover in these preparations (Goldberg, 1980). Interestingly, during fasting, leucine, like fatty acids or ketone bodies, serves as an alternate substrate to glucose and is the only amino acid that can provide significant energy for muscle (Chang and Goldberg, 1978).

Catecholamines: Epinephrine, at physiological concentrations, reduces proteolysis in skeletal muscle while norepinephrine also causes a nett reduction in degradation, but not to the same degree (Garber et al, 1976). This suggests that the effect on protein degradation is more a β adrenergic effect and this has been confirmed since a β agonist (isoproterenol) but not an α-agonist (phenylephrine) can mimic the response to epinephrine.

Contractile activity of muscle: The addition of glucose, insulin as well as amino acid, leucine to intact isolated muscle did not prevent a nett state of degradation in this *in vitro* system. As these muscles differ in one further respect,with regard to *in vivo* muscle in that they do not perform work, the effect of repetitive stimulation and passive tension on protein turnover *in vitro* has been studied (Goldberg *et al*, 1980). Both these factors retard protein breakdown, so that if isolated muscles are provided with the above-mentioned factors and maintained under some degree of tension, neutral or even positive nitrogen balance *in vitro* can be observed.

1.3.2. Influence of various factors on protein turnover measured *in vitro*

Pituitary and thyroid hormones. The observation that hypophysectomy of young animals prevents normal body growth and protein accumulation in skeletal muscles is well known (Flaim *et al*, 1978a). Goldberg and co-workers (1980[b]) confirmed earlier findings that protein synthesis is reduced following the removal of the pituitary, but in addition, showed that rates of protein breakdown were also much slower in muscles of hypophysectomized rats. The fall in protein synthesis was attributed to the lack of growth hormone, while the reduction in protein catabolism could not be immediately explained. Treatment of hypophysectomized rats with growth hormone did not alter rates of protein degradation,

while rates of protein synthesis increased (Goldberg, 1969; Goldberg et al, 1980b). Further studies indicated that deficiency of thyroid hormones was responsible for the decrease in protein breakdown following hypophysectomy (Flaim et al, 1978b). Treatment of these rats with either triiodothyronine (T_3) or thyroxine (T_4) increased the rates of protein degradation to levels seen in control animals (Goldberg et al, 1980b). This stimulation of proteolysis by thyroid hormones was similarly observed in thyroidectomized rats, which also had decreased levels of protein degradative rates. Treatment of these rats with T_3/T_4 also stimulated protein synthesis in muscle.

The same group of investigators also noted that small doses of T_4/T_3 to hypophysectomized rats stimulated synthesis more than breakdown (anabolic), whereas administration of these hormones in excessive amounts resulted in enhanced protein breakdown and loss of weight (catabolic effect). They thus demonstrated that a minimal level of thyroid hormone is essential for normal growth, while an excess of the same (hyperthyroidism) leads to a general loss of weight and severe muscle wasting. An intriguing and yet unexplained question is how this hormone can have different dose-response curves in a single tissue for its effects in stimulating protein synthesis and degradation.

Glucocorticoids: It is well known that the over-production of adrenal steroids in Cushing's Syndrome leads to marked wasting of muscle and loss of strength. Although generally

assumed to promote protein breakdown, the effects of glucocorticoids on protein turnover in muscle remain controversial. Early studies indicated that cortisol stimulated protein breakdown (Goldberg, 1969[b]). However, several studies have not only failed to confirm glucocorticoid-induced enhanced proteolysis but furthermore, their observations indicate a reduction in rates of both protein synthesis as well as protein degradation,by glucocorticoid administration (Shoji and Pennington, 1977; McGrath and Goldspink, 1980). However, this reduction affected protein synthesis more than protein degradation, with a nett increase in protein degradation, although the absolute values of protein degradation tended to be lower than controls. Studies with adrenalectomized rats have shown that fasting produced little, if any, nett increase in proteolysis in muscle, while muscle from fasted control rats revealed the well known effects of decreased protein synthesis and enhanced degradative rates. Administration of glucocorticoids to the fasted, adrenalectomizedrats resulted in increase in proteolytic rates similar to the fasted control animals (Goldberg, 1980). These experiments demonstrate a clear requirement for glucocorticoids for the acceleration of protein breakdown during fasting. On the other hand, when fed rats (normal/adrenalectomized) were treated with glucocorticoids no increase in muscle protein breakdown was observed, suggesting that some additional hormone which is dependent upon nutritional status (e.g. insulin) may be important in the response of muscle to steroids.

An interesting observation by Santidrian et al (1981) concerns an elevated urinary output of 3 methyl histidine associated with cessation of growth in adrenalectomized rats treated subcutaneously with large doses of corticosterone. Intraperitoneal administration of the glucocorticoid resulted in much less severe retardation of growth and gave no changes in 3-methylhistidine output. This suggests that the intraperitoneal route of administration was accompanied by hepatic inactivation of the corticosteroid.

Starvation: Food intake and food deprivation (starvation) are associated with a rise and fall in insulin levels respectively, the former situations characterized by increased protein synthesis and decreased proteolysis, whereas the latter by a fall in protein synthesis and a nett release of amino acids from muscle. These effects confirm the role of insulin in growth and its importance in maintaining positive nitrogen balance in muscles (Cahill, 1970; Felig, 1975).

Disuse/Denervation Atrophy: Disuse and denervation lead to marked atrophy of muscle which is mainly due to enhanced proteolysis (Goldberg, 1969b; Goldspink, 1976). The decrease in rates of protein degradation obtained when isolated muscles are maintained under passive tension lends support to the argument by physiotherapists that passive exercise of disused muscles retards the degree of atrophy (Goldberg,1979). Du Bois and Almon (1981) have detected increase in the number of glucocorticoid receptors in denervated compared with contralateral control gastrocnemic muscles, supporting the conclusion

that such atrophy could be the result of increased sensitivity of individual muscles to normal levels of glucocorticoids. These results demonstrated that the increase in glucocorticoid receptor sites preceded atrophy, ruling out the possibility of a concentration effect. In addition, Du Bois and Almon (1981) have shown similar increases in glucocorticoid receptors in muscles undergoing disuse atrophy. That denervation and disuse atrophy may be mediated by enhanced sensitivity to normal levels of hormones, while innervation may play a role in regulating such responsiveness seems an attractive mechanism, although further clarification of the biochemical details underlying such a mechanism is required.

Testosterone: It has been known for some time that castration of rats is followed by decreased growth rate of all skeletal muscles as well as reduced body growth. These levels are restored to normal by testosterone administration, thus confirming the important anabolic effect of androgens (Scow, 1952). Although the anabolic effects of this hormone are well established, little information exists as to its influence on protein degradation. The increase in muscle mass which follows testosterone treatment in castrated animals suggests that in the absence of testosterone, protein degradation in muscle is not retarded. Dahlmann and co-workers (1980) have studied the effects of castration and subsequent administration of testosterone on the activity of an alkaline protease in skeletal muscle, they demonstrated a 2-3 fold increase in specific

activity of the alkaline protease in the castrated group, compared with controls; normal levels were attained on testosterone treatment. In another report, they have described a 50% increase in alkaline protease activity in EDL muscles of rats undergoing endurance training and this exercise-mediated increase was prevented by the simultaneous administration of testosterone (Dahlmann et al, 1981[a]). They conclude that testosterone has a regulatory effect on the protein degradative mechanism - although examination of the method of preparation of the muscle extract reveals a high salt buffer system, ideal conditions for solubilizing the mast cell chymase (Pastan and Almqvist, 1966); hence, the activity studied was almost certainly the non-muscle cell entity. Evidence for the role of non-muscle cell proteases in the degradation of muscle cell proteins is not available and therefore the significance of such correlations in relation to the present knowledge with regard to models for the intracellular degradation of muscle-cell derived proteins is not known.

Mechanism(s) of hormonal control: It is therefore clear from the above studies that overall rates of protein degradation in muscle, like protein synthesis, are subject to precise hormonal control. However, the biochemical details of the fine mechanism of this control are at this stage mainly speculative. Investigators in this field have looked at the activity levels of muscle proteases in various muscle disease states to determine whether a correlation between muscle wasting and protease activity could be demonstrated. The

report by Dahlmann and co-workers mentioned above is such an example. Subsequently to these studies, Dahlmann et al (1981) have also reported that the increased alkaline protease activity in starvation, diabetes, testosterone deficiency, glucocorticoid injected rats and muscle dystrophy suggest an involvement of the mast cell chymase in these pathological states, although they conclude that with mast cell degranulation (which abolishes chymase activity) these muscles still undergo increased catabolism indicating that 'other proteases' (and not the mast cell chymase) are responsible for the increased degradation. Toyo-Oka (1980) has studied the activity levels of Cathepsin B, Cathepsin D and calcium activated neutral protease in cardiac and skeletal muscles of hyperthyroid animals and showed that while all these activities were increased in skeletal muscle extracts of the hormone-treated animals, the activities remained unchanged in cardiac muscle extracts. De Martino and Goldberg (1978) reported a similar increase in the content of lysosomal enzymes in liver and skeletal muscle induced by administered thyroid hormone. Studies by Hoh et al (1977), Sartore et al (1981) and more recently by Chizzonite et al (1982) have indicated that addition of thyroid hormone results in isomyosin transformation in the heart and these data provide a molecular basis for the observed effects of thyroid hormone on the heart (i.e. hypertrophy, increased myofibre-shortening velocity and significant increase in calcium-stimulated myosin ATP-ase activity). Mayer and Shafrir (1974) measured adaptive changes of "rat myofibrillar protease" activities in rats after gluco-

corticoid administration, alloxan-induced diabetes mellitus, aminonucleoside induced nephrotic syndrome, fasting, and hypoproteinaemia induced by plasmapheresis. The specific activity of the protease wasincreased in all these adaptive states, and although this protease at the time of the report was thought to be of muscle-cell origin, this entity was almost certainly the mast cell chymase. Katanuma and Kominami (1977) have suggested a physiological role for the group-specific protease for apoproteins of pyridoxine enzymes, which also increased in states of starvation and with age. However, this entity once more represented mast cell chymase and not muscle-cell derived protease activity.

Therefore, although cell-free biochemical experimentation may provide data in support of a physiological role for several lysosomal and non-lysosomal protease, in many cases the cellular location of the protease under study has not been verified, while in other cases the effects of the presence of endogenous inhibitors, compartmentalization, activation/inactivation of proteases, make the definition of the details of such hormonally mediated effects complicated.

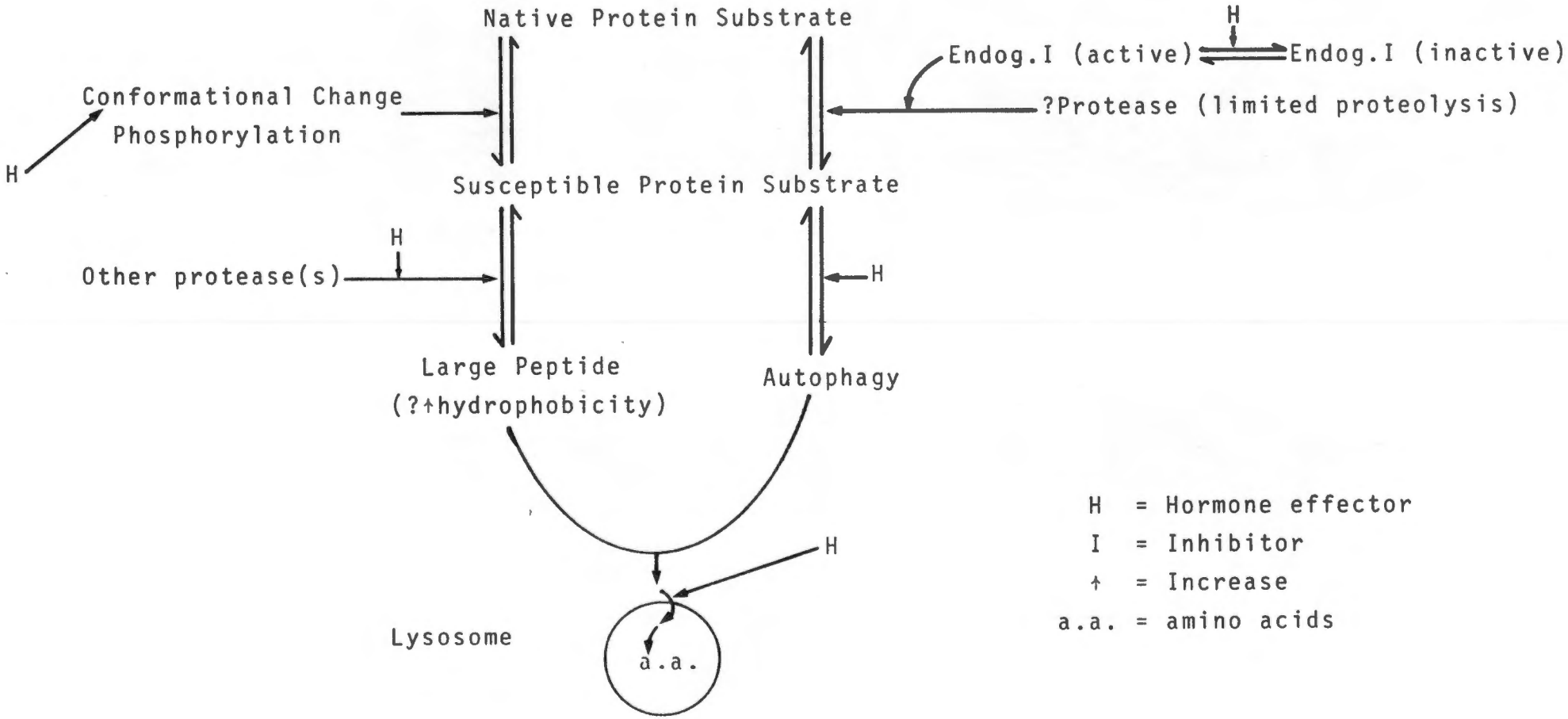
As mentioned earlier, the fact that nutritional step-down and some hormonal effects appear to be mediated via autophage lysosomal pathways, the findings of enhanced activities of lysosomal proteases following the in vivo manipulation of hormonal status support these observations. However, consideration of the pathway models for the degradation of intra-

cellular proteins suggest that the rate-limiting steps are likely to be at the level of substrate susceptibility, early limited proteolytic events or the stage of membrane attachment (uptake by lysosomes) and not at the level of lysosomal protease content, as the latter step would require selective recycling of native cytosolic material after its entry into lysosomes - and as such recycling has not been demonstrated and is unlikely to return native functional cytosolic proteins unaltered, selectivity of degradation at the intra-lysosomal stage of protein degradation appears unlikely. Thus at present, in terms of the degradative model, the rate-limiting event(s) for the hormonal regulation of protein degradation appear(s) to have an extra-lysosomal location. Such possible sites are outlined schematically (Fig. 1.5), where hormonal regulating factors may act at various rate-limiting steps of the pathway, while other proteases (lysosomal and non-lysosomal) may also participate in this process though not limiting the overall process.

The importance of hormonal factors in the influence and control of protein degradation is clear, although the molecular mechanism(s) underlying these events are not characterized at this stage. Further insight into the knowledge of the physiologic pathway(s) of protein degradation ought to shed more light on this regulatory mechanism.

Fig. 1.5

INTRACELLULAR PROTEOLYSIS: REACTION STEP(S) THAT MAY BE UNDER ENDOCRINE CONTROL



1.4. METHODOLOGIC CONSIDERATIONS

The technical problems involved in measuring rates of proteolysis in muscle are particularly challenging. Three experimental approaches have generally been used to study intracellular protein degradation. The first has attempted to measure degradation rates *in vivo* by administration of a pulse of a radio-labelled amino acid to serve as a precursor for incorporation into protein in the whole animal. The rate of release of the label from the polymeric form back into the free amino acid pool is then considered to be a measure of degradation. Calculation of rates of protein breakdown in muscles of intact animals (*in vivo*) involve a number of assumptions that are still controversial; such calculations are also subject to a number of artifacts, such as those due to re-utilization of radio-isotopically labelled amino acids (Goldberg and Dice, 1974; Zak *et al*, 1977).

The second experimental approach has been the use of isolated cells or cells in culture. This technique allows easy access to control of environmental conditions, e.g. additions of hormones, amino acid depletion, lack of vitamins, effects of protease-specific inhibitors, energy depletion, etc. - thus providing information on the behaviour of the entire degradative machinery and indicating to what degree inhibitors of known proteases affect this process. For example, the different rates of degradation obtained from endogenous but extracellular ^{3}H-labelled macrophage proteins and the endogenous but intracellular ^{14}C-labelled proteins, as well as the

observation that the breakdown of the ^{3}H-labelled proteins was more strongly inhibited by chloroquine, suggested that different mechanisms in different compartments of the cell were responsible for the degradation of exogenous and endogenous proteins (Kay, 1978).

The urinary excretion rate of 3-methyl histidine has been used as an index of muscle-protein degradation in vivo (Young et al, 1972; Long et al, 1977; Ballard et al, 1979). This amino acid originates from the degradation of actin and some species of myosin heavy chains in which it is present after the post-translational methylation of specific histidine residues (Young et al, 1972). Following degradation of myofibrillar protein, the methylated histidine is released, does not undergo re-utilization or metabolism, and is quantitatively eliminated in the urine (Young and Munro, 1980). The use of 3-methyl histidine as a measure of muscle protein breakdown rate assumes that the bulk of urinary 3-methyl histidine comes from skeletal muscle, with only a small proportion being released from other organs, notably skin and intestines (a meat-free diet is also required). In this regard, measurement of 3-methyl histidine turnover in rat skeletal muscle, skin and gastrointestinal muscle, indicated that skeletal muscle contributed to only 25% of the total urinary excretion (Millward et al, 1980). However, Harris (1981) has shown, by recalculating data of urinary 3-methyl histidine excretion in rats, that skeletal muscle tissue is the major contributor of 3-methyl histidine in the urine, a conclusion supported by Santidrian et al (1981).

Thirdly, in order to measure protein degradation under carefully controlled conditions, wide use has been made of a relatively simple and standardized *in vitro* techniques involving the use of thin rat or mouse muscles that can be maintained *in vitro* for several hours in an apparently stable physiological state (e.g. the diaphragm, red soleus muscle, or pale extensor digitorum longus muscle) (Fulks *et al*, 1975).

Overall protein breakdown in such isolated muscles is measured by determining the release of tyrosine from tissue protein. Nett production of this amino acid must represent a nett protein breakdown by the tissue since muscle neither synthesizes nor degrades tyrosine. However, some investigators have equated protein breakdown with the release of alanine and glutamine from muscle - this is not valid as these amino acids are synthesized *de novo* in large amounts (Garber *et al*, 1976; Odessey *et al*, 1974). Since these tissues *in vitro* generally maintain constant intracellular pools of tyrosine, the release of tyrosine from muscles reflects the nett hydrolysis of tissue protein, that is the rate of tyrosine production by protein breakdown minus the rate of tyrosine re-incorporation through protein synthesis. To measure protein breakdown in isolation, muscles have generally been incubated in the presence of cycloheximide (0,5 mM) which inhibits protein synthesis (Fulks, *et al*, 1975; Libby and Goldberg, 1980). Although cycloheximide may reduce absolute rates of degradation, the regulatory effects of various physiological factors on proteolysis can still be measured (Fulks

et al, 1975). Where this has been studied, the rates of protein breakdown in intact animals have correlated well with the rates of this process in isolated muscles, e.g. in hypophysectomized animals treated with replacement doses of thyroid hormone, the rate of muscle protein breakdown increases as measured both in vivo, using a pulse chase technique, or in the isolated muscles in vitro (De Martino et al, 1977).

Further useful contributions from cell-free studies in vitro include cell-free experimentation which has led to the identification of several lysosomal and non-lysosomal proteases as well as endogenous inhibitors. As muscle tissues contain cells of many types (i.e. myocytes, fibroblasts, mast cells, endothelial cells, adipocytes), mixing of subcellular fractions from these differing cell types cannot be avoided. One such non-muscle cell which populates muscle tissues and contains vesicles packed with "alkali" and "acid" hydrolases, is the mast cell (Pepys and Edwards, 1979). This fact has recently led to investigators ridding muscles and other tissues of these cells by mast cell degranulation, prior to evaluating in vitro muscle protein degradation in intact muscle (Libby and Goldberg, 1980) or cell-free protease activities in muscle homogenates (McKee et al, 1979; Edmunds and Pennington, 1981).

In addition, it should be borne in mind that intravascular components may also provide tissue homogenates to some extent with their accompanying proteinases, e.g. serine proteinase

of neutrophil granulocytes (Barrett, 1977[a]) as well as plasma proteinase inhibitors such as α_2 macroglobulin, α_1 protease inhibitor and α_1 chymotrypsin (Koj and Kurdowska, 1981). Although after having stated this, it ought to be mentioned that Stauber *et al* (1981) have shown by immunohistochemical techniques that the protease inhibitors α_1 antitrypsin and α_1 inhibitor$_3$ are located within muscle cells in normal rats, and this intracellular pool decreases in states of enhanced proteolysis such as experimental diabetes mellitus - providing a model whereby extracellular protease inhibitors modulate intracellular degradative events (e.g. autophagy), and the constant supply of inhibitor (possibly by endocytosis) could be retarded under nutritional step-down and other physiologic or pathologic states.

Findings with cell-free preparations cannot prove the physiologic function of a protease or inhibitor *in vivo*, as differences between enzyme behaviour in intact cells and in cell-free preparations may result from: release of endogenous inhibitors, lack of appropriate *in vitro* conditions, cellular or subcellular compartmentation of the enzyme. Nevertheless, Hershko and co-investigators have characterized the above-described multicomponent ATP-dependent proteolytic pathway in cell-free extracts which, for the first time, offers a mechanistic explanation for the previously well-known energy requirement of protein degradation. Such observations of the behaviour of cell-free components *in vitro*, which are in accord with *in vivo* properties, argue strongly for the use of

such isolation and subsequent reconstitution studies of various subcellular components as a vital tool towards a total understanding of the overall degradative process. Furthermore, the observations of the effects of endogenous and exogenous inhibitors on the activities of purified proteases have been extended to a study of these inhibitor effects on intact isolated muscles (Libby and Goldberg, 1980) as well as on cells in culture (Dean, 1980), with a view of examining the roles of these proteases in in situ and in vivo degradation and an ultimate aim of understanding the pathway(s) of protein degradation and its therapeutic manipulation in various muscle disease states.

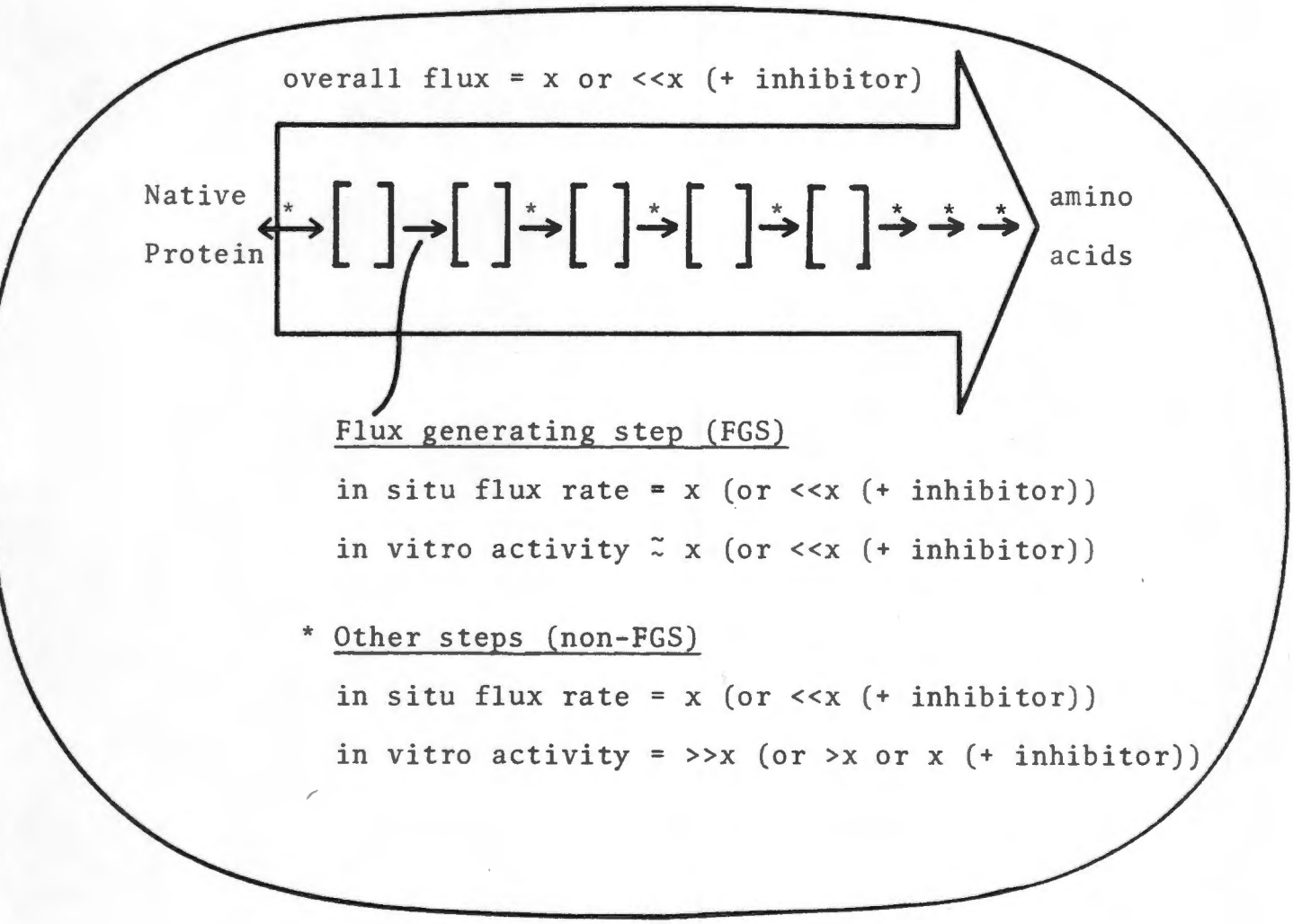
Degradation studies with intact muscle tissue, isolated muscle cells and muscle cells in culture provide information about the in situ behaviour of the proteolytic machinery as a functioning unit. Thus, manipulation of conditions in vivo or in vitro which alter the flux of the entire system would be expected to be acting primarily (but not exclusively) at the site(s) of the rate-limiting step(s) for the overall process(es). However, since precise biochemical details about the individual processes and steps in the degradative pathway are not available at present, caution must be exercised in the interpretation of such studies. Cell-free studies, although associated with certain limitations (as described above), provide the means towards the identification of subcellular constituents and participitants in protein degradation process(es) (i.e. substrates (state, availability and susceptibility), proteases; inhibitors; cofactors), and therefore, correlations between

assayable levels of proteases (or inhibitors) with whole organ proteolytic rates in states of altered proteolysis may provide evidence that such entities operate at flux-generating steps (FGS) in the metabolic process(es) of protein degradation (Fig. 1.6). The corollary to this is that cellular components that show levels of assayable activities which do not follow the overall direction of the protein breakdown process *in situ*, while not being candidates for consideration as rate-generating components, could very well be participants in the degradative pathway at *non*-regulatory steps (non-FGS), especially if their location is demonstrated to be within muscle cells (Fig. 1.6).

These flux-generating proteolytic events could mechanistically be mediated via one of several possibilities: absolute changes in protease levels; alterations in inhibitor content (interconversion from active to inactive forms or vice versa); alternatively, pinocytosis of extracellular plasma inhibitors such as α_1 antitrypsin which may then influence intracellular processes; changes in intracellular milieu (e.g. ionic strength, pH) which may suit a specific inhibitor, protease or cofactor; or alterations in substrate susceptibility to a particular protease. In such a scheme (Fig. 1.6), the rate-limiting step may be controlled by regulatory factors (e.g. hormones or inhibitors), while other non-limiting components carry out their relevant functions in the face of no (or even paradoxical) changes in their assayable activities.

Fig. 1.6

MODEL FOR FLUX-GENERATING STEP:-



Thus, whereas experiments carried out *in vivo* provide data on rates of degradation of individual proteins and alterations in response to perturbation of some sort, the molecular mechanisms, properties of intracellular proteases, substrate susceptibility and effects of endogenous inhibitors remain uncharacterized in such an experimental design. In contrast, adoption of the approach utilizing systems *in vitro* provide the molecular details of individual proteases, endogenous inhibitors, and other subcellular participitants in degradation and possibly even their regulation, and although one may argue that this may not directly represent the functions of these entities *in vivo*, by combining the information gained by the different experimental strategies, investigators are arriving closer at a composite picture of the process of intracellular protein degradation.

1.5. OBJECTIVES OF THIS STUDY

Investigations into muscle protein degradation, more specifically in relation to rat skeletal muscle, have provided more and more information pointing to possible roles played by intracellular proteases and endogenous inhibitors in intracellular proteolysis. In addition, regulation by hormones appears to be complex, while the energy requirement of the overall process of protein degradation to the level of amino acids, requires an explanation.

Particularly interesting questions that arise out of these considerations include: Can the components of intracellular pathways of protein degradation be reconstituted by the classical biochemical approach of resolving the complex system by separation procedures, analysing their individual activity and assessing how they interact upon reconstitution? Is there a specific myofibril-associated protease in muscle cells? Does the activity of the mast cell-derived enzyme chymase alter when *in situ* proteolytic rates in skeletal muscles alter? Does the state of substrates or enzymes affect their interactions with endogenous inhibitors? Can the effects of various hormonal or other manipulations on the overall catabolic process in isolated skeletal muscles be correlated with changes in the assayable levels of specific, possibly rate-limiting enzymes or inhibitor levels? Can the exciting observations of a multi-component ATP-dependent proteolytic system in reticulocytes be extended to skeletal muscles, indicating a general role for such a system in protein degradation?

Attempts to answer these questions formed the basis of the work reported in this thesis. The means employed included cell-free and intact tissue studies of skeletal muscles derived from laboratory rats.

CHAPTER 2

THE PROPERTIES AND SIGNIFICANCE OF THE MYOFIBRIL-ASSOCIATED PROTEINASE IN RAT SKELETAL MUSCLE HOMOGENATES

2.1. INTRODUCTION

There has recently been considerable disillusionment amongst workers in the field of protein degradation, with the most active protease whose activity can be detected at mildly alkaline pH in skeletal muscle homogenates (Noguchi and Kandatsu, 1970; Yasogawa et al, 1978; Koszalka and Miller, 1960; Reinauer and Dahlmar, 1979). This arises from the fact that there is convincing evidence that the "myofibrillar alkaline protease" has its origin in the mast cells which populate rat skeletal, heart and smooth muscle tissue in small but significant numbers (Woodbury et al, 1978; McKee et al, 1979; Libby and Goldberg, 1980). Accordingly, there is now more or less general agreement that the chymostatin-sensitive serine protease which associates with the particulate fraction of muscle homogenates and which destroys a variety of protein substrates, including the heavy chains of myosin, is "chymase" present in the granules of the mast cells and liberated during homogenization because of the fragility of the particles (Edmunds and Pennington, 1981; Reinauer and Dahlmann, 1979; McKee et al, 1979).

At the present time, the questions which remain are whether there exists a true muscle-cell derived myofibrillar protease, and why the mast cell protease adheres to myofibrils with such tenacity that its purification free of myofibrillar proteins is a difficult problem when the starting material is washed myofibrils (Bosch, 1981). In addition, the studies of Mayer et al (1974) have suggested that the chymase activity of

skeletal muscles in starved, diabetic and cortisol-treated rats varies in a manner consistent with the enzyme's involvement in rate-limiting steps in protein degradation. No quantitative studies on mast cells in such rat tissues are currently available.

At a broader level, the more challenging question that remains unanswered is the reconstitution and resulting characterization of the step-wise pathway by which selective degradation of susceptible proteins, to the level of amino acids, occurs in living muscle cells. Despite the isolation and detailed characterization of various proteases in muscle homogenates (see Chapter 1), there is as yet no well-supported model for proteolysis pathways in which specific roles can be assigned to any or all of the various proteases and their endogenous inhibitors. It is particularly in relation to the latter proteins that there is a shortage of ideas as to how the balance between proteases and inhibitors is achieved in the context of highly complex intracellular structural organization in which some proteins are soluble and others membranous or sequestered in cellular compartments.

Rat skeletal muscle homogenates have accordingly been investigated by the use of differential centrifugation to prepare subcellular fractions. Protease activities present in the fractions, as well as enzyme-substrate and enzyme-substrate-inhibitor interactions, were examined in single and in reconstituted fractions, in an attempt to clarify the contribution of different subcellular compartments to the overall proteo-

lytic process, and to examine the possible ways in which inhibitors operate. The presence of a myofibril-associated protease (chymase), active at pH 7,4 and above, has been confirmed in such preparations. An interesting difference in the response of filamentous and soluble 'chymase' to inhibitors present in cytosol fractions has been observed. This may provide a model for the differential control of cellular proteinases by inhibitors whose effectiveness can be modulated by variable supramolecular structure.

2.2. MATERIALS AND METHODS

2.2.1. Preparation of rats

Long-Evans hooded rats of both sexes (100 or 200 gram body mass, as specified in the text and legends) were injected intraperitoneally with an anaesthetic dose of pentobarbitone sodium and sacrificed by decapitation. The rats were maintained, before experimentation, on standard laboratory chow.

2.2.2. Subcellular fractions from rat diaphragm

Diaphragms were carefully dissected out, freed of fat and connective tissue and cut into fine pieces. These were homogenized with an Ultraturrax motor-driven homogenizer (Janke and Kunkel, FRG), using two 30-second bursts at medium speed, with cooling. Five millilitres of Buffer A (i.e. 25 mM tris-HCl, 0,1 M KCl, 2 mM $MgCl_2$, 2 mM sodium pyrophosphate, 2 mM EDTA, and 1 mM dithiothreitol (DTT) pH 7,4) were used per gram of tissue. The homogenate was centrifuged at 800 g

for 15 minutes; the supernatant was saved (see below) and the pelleted material was taken up (5 ml per gram original material) in Buffer B (i.e. Buffer A minus pyrophosphate), before being resuspended and washed by re-centrifugation.

The washed pellet was now treated with Buffer B, to which triton-X 100 was added to a final concentration of 1%. After centrifugation as before, three further washes in triton-X 100-free buffer were performed (a modification of a procedure by Etlinger et al (1976)) and the purified myofibrils (MF), which contained no other elements on light microscopic examination, were taken up in 0,6 M KCl.

The supernatant from the first centrifugation step described above (apart from samples retained for assays), was centrifuged in a Beckman J-21 refrigerated centrifuge at 20 000 g for 15 minutes, to obtain a mitochondrial/lysosomal pellet (ML) (taken up in 5 ml of Buffer B) and a supernatant fraction; the bulk of the latter was recentrifuged at 100 000 g for 60 minutes. The microsomal pellet (SR/SL) thus obtained was also resuspended as described above, and the soluble fraction was termed the cytosol fraction. All procedures were carried out at 0-4^{o}C.

2.2.3. Protease Activities

2.2.3.1. Determination of activities in the various subcellular fractions and reconstitution studies at pH 7,4

The subcellular fractions thus obtained were incubated, individually at first, for 3 h at 37°C, in a buffer containing 25 mM tris-HCl, 0.1 M KCl (or 0.6 M KCl where specified), 2 mM EGTA, 1 mM DTT, 2 mM $MgCl_2$ (all at pH 7,4), in the absence and presence of added azocasein (2 mg). The incubations were terminated by addition of trichloroacetic acid (TCA, 5% final concentration). Aliquots of the TCA-soluble supernatants were assayed fluorimetrically for their content of free tyrosine or tyrosine peptides, according to the method of Waalkes and Udenfriend (1957). Protease activity was expressed as nmoles tyrosine/mg protein (type specified)/3 h. Thus 'MF protease activity' was expressed as nmoles tyrosine/mg MF protein/3 h.

In addition to the measurement of individual protease activities in the various subcellular fractions (with either endogenous (autolysis) or exogenous substrates), the fractions were also incubated in various combinations to determine whether complementation between them could be established on reconstitution. Appropriate zero-time controls were used throughout. Protein was measured by the modified method of Lowry *et al* (1951) with bovine serum albumin as standard.

The TCA-precipitable myofibrillar proteins were subjected to SDS-polyacrylamide gel electrophoresis (in a 10% polyacrylamide slab gel) (Porzio and Pearson, 1977), to detect higher molecular weight degradation products of individual myofibrillar proteins.

2.2.3.2. Activities of "myofibril-associated protease" and calcium-activated protease in various muscle fibre types

Myofibrillar (MF) and post-mitochondrial lysosomal supernatant (PMLS) fractions were prepared from various rat muscles (some containing predominantly the fast-twitch, white muscle fibres and others predominantly slow-twitch red fibres).

"Myofibril-associated protease" (MF-protease) activities were measured in the MF fractions at pH 7,4 and 37^{o}C for 3 h (high salt - 0,6 M KCl) in the buffer conditions described in 2.2.3.1. above. MF-protease activity was measured by determination of solubilized tyrosine or tyrosine peptides and expressed also as in 2.2.3.1. above.

Calcium-activated protease (CAP) activities in the PMLS fractions were measured as described by (Pennington, 1977), after removal of "CAP inhibitor" by acid pre-treatment (pH 5).

The various muscles studied in this manner included diaphragm (mixed red and white muscle fibre type); soleus (predominantly red); extensor digitorum longus (white); psoas (white), gluteus medius (white) and cardiac ventricular muscle (Millward

et al, 1978).

Activities were expressed as nmoles tyrosine/mg protein/3 h.

2.2.4. Partial purification of protease

A triton-washed myofibrillar fraction was solubilized in 0.6 M KCl containing 25 mM tris-HCl, 1 mM DTT and 1 mM ATP (pH 7.4). The soluble material (2 ml) was chromatographed on a Sepharose 6B column (0.9 x 60 cm) and fractions of 1 ml were assayed for protease activity in the presence of added azocasein. The fractions containing the protease were pooled and assayed with azocasein or cytosol (same protein concentration), at both low and high ionic strength (0.15 and 0.6 M KCl, respectively). In addition, the protease-containing fraction was dialysed for 12 h against a buffer at 0.15 M, centrifuged at 20 000 g for 20 minutes, and the resultant supernatant and pellet both re-assayed to determine the extent to which the protease activity was sedimentable at low ionic strength in the absence of the bulk of myofibrillar proteins (viz. actomyosin).

2.2.5. Skeletal muscle cells

The established myoblast cell line L8 was originally isolated from rat skeletal muscle (Richter and Yaffe, 1970). Cells were grown at $37^{o}C$ in a humidified incubator with 5% CO_2 in air. After myoblast proliferation to confluence in the stock flask, the cells were dissociated with trypsin-EDTA solution and seeded at 10^5 cells per 35 mm petri-dish containing 2 ml of growth medium. Once confluent, the cells progressively

fused to form large multinucleate syncytia (myotubes) which synthesized muscle-specific proteins and contracted spontaneously after 7-8 days' growth. Cells were used between fourth and tenth passage in culture, and all experiments were initiated on dishes showing approximately 80% of nuclei in myotubes, i.e. 80% fusion (7-8 days' growth). Cell numbers and percentage fusion were determined by counting nuclei in cultures fixed in methanol and stained with 0.25% May Grunwald for 10 minutes, followed by 10% Giemsa for 20 minutes. Skeletal muscle cells were harvested from the dish by scraping into Buffer A (see above) with a rubber policeman. The cell samples were then homogenized and the MF fraction washed and prepared as described above. The washed MF pellet was suspended in Buffer B (lacking sodium pyrophosphate) and this material was assayed for protease activity, with and without casein, at low and high salt conditions as described above. Similarly, SDS-polyacrylamide gel electrophoretic analysis of the MF fraction before and after incubations was performed to determine the major protein constituents of this MF fraction from skeletal muscle cells and in addition to detect higher molecular weight degradation products.

2.2.6. Mast cell degranulation

Degranulation of mast cells was induced according to the method of Pastan and Almqvist (1966) and Riley (1959). Rats weighing about 200 grams received bi-daily intraperitoneal injections of compound 48/80 (Sigma Chemical Co.), according to the following protocol: Day 1 - 100 µg/100 gm; Day 2 -

200 µg/100 gm; Day 3 - 300 µg/100 gm; Day 4 - 400 µg/100 gm; Day 5 - 500 µg/100 gm. The rats were sacrificed on Day 6, and tne diaphragms and selected leg muscles were dissected out for fractionation by differential centrifugation, for incubation as described above and for histological studies.

2.2.7. Preparation of radiolabelled substrates

[^{14}C methyl]globin was prepared by labelling bovine haemoglobin according to the method of Price and Means (1971), followed by haem extraction as described by Schapira et al (1968).

^{14}C-N-ethylmaleimide-labelled myofibrillar proteins (5 x 10^5 dpm/mg) were prepared using myofibrils derived from skeletal muscles freed of mast cells by prior treatment with compound 48/80 (see above). Triton-washed myofibrils were obtained as described under 'Subcellular fractions from rat diaphragm', solubilized in 0,6 M KCl, 50 mM Tris-HCl, pH 7,4 (lacking DTT) and labelled with ^{14}C-N-ethylmaleimide (^{14}C-NEM) (10 mCi/mmol) generally as described by Gerard and Schneider (1979): 8 µCi of ^{14}C-NEM was added to 5 mg of myofibrillar protein to give a final NEM concentration of 1 mM. After incubation for 30 minutes in the high-salt buffer system, five volumes of ice-cold distilled water were added and the myofibrils washed three times by repeated centrifugation in this manner. Thereafter the myofibrils were suspended in 50 mM Tris-HCl, 0,1 M KCl, 2 mM $MgCl_2$, 1 mM DTT and dialysed in the cold against this buffer before use.

2.2.8. Protease assays with labelled protein substrates

Each assay contained 50 mM Tris HCl, 0,6 M KCl, 2 mM EGTA, 1 mM DTT, 2 mM $MgCl_2$ (all at pH 7,4), together with 1 mg MF fraction and radioactive substrate (1,5 x 10^4 dpm in the case of [^{14}C methyl]-globin or 8 x 10^3 dpm of ^{14}C NEM-myofibrils), in a final volume of 250 µl. Incubations were performed at 37°C for 60 min, after which 200 µl 10% ice-cold trichloroacetic acid (TCA) containing 5 mg/ml carrier bovine serum albumin was added to each to stop the reactions. Aliquots (200 µl) of the supernatant fractions were mixed with 5 ml Instagel (Packard Instrument Co., Downer's Grove, Illinois, USA) and counted in a Beckman LS 9000 Liquid Scintillation Counter. Protease activity was expressed as dpm released in acid-soluble form per hour, after subtraction of values for reactions not containing added enzyme.

2.2.9. Materials

Dithiothreitol (DTT), EGTA, Compound 48/80 and L-triiodothyronine were obtained from Sigma Chemical Co. [^{14}C] Formaldehyde (16.7 mCi/mmol) and [^{14}C]N-ethylmaleimide (10 mCi/mmol) were obtained from Amersham, England. Chymostatin, leupeptin and pepstatin were purchased from the Peptide Institute, Protein Research Foundation, Japan. All other chemicals used were of Analytical Reagent grade.

2.3. RESULTS

Various subcellular fractions, derived from rat diaphragms, were incubated either singly (autolysis) or in the presence of added azocasein, in order to assess their respective protease activities. Only the myofibrillar fraction (MF) released significant amounts of tyrosine in the presence and absence of added azocasein, under conditions of ionic strength = 0.150, i.e. in four separate experiments, one milligram of MF released 11, 12, 10 and 11.5 nmoles of tyrosine per 3 h, while with added azocasein (2 milligrams), these rates were increased to 26, 28, 25 and 30 nmoles tyrosine/3 h, respectively. Thus the MF contained both enzyme (protease(s)) and susceptible substrate(s). Triton washing and repeated reprecipitations from high salt solution of MF caused no fall in the specific activity, suggesting either that the protease had solubility properties very similar to those of actomyosin or that the enzyme was associated tightly with these contractile proteins at low ionic strength, within the native myofibrillar form or in "natural actomyosin" (filaments).

Complementation tests were next carried out on the subcellular fractions from rat diaphragms. In all combinations between MF and cytosol-containing fractions, an enhancement of proteolysis beyond the expected additive effects was observed (Table 2.1).

This complementation suggested that there was either (a) an activator of the "MF protease" in the soluble fraction, or (b) that a protease in the cytosol acted well on MF proteins,

TABLE 2.1

COMPLEMENTATION IN PROTEOLYSIS BETWEEN MYOFIBRILLAR AND CYTOSOLIC FRACTIONS

Aliquots of MF fraction (each containing 1 mg protein) were incubated independently and in the presence of constant amounts (in mg protein) of the various subcellular fractions - the assays and expression of protease activity were as outlined in Methods. The same results were obtained in three separate experiments.

	PROTEOLYSIS (nmoles tyrosine/mg/3 h
MF	10
Post MF supernatant	<0,1
MF + Post MF supernatant	26
Post ML supernatant	<0,1
MF + Post ML supernatant	25
ML	<0,1
MF + ML	10
Cytosol	<0,1
MF + Cytosol	27
SR/SL	<0,1
MF + SR/SL	10,5

as substrates, or (c) that the "MF protease" acted particularly well on added soluble substrates available in the cytosol.

Possibility (b) was rendered unlikely by the absence of azocaseinase activity in the cytosol (Table 2.2). The other two possibilities were accordingly further investigated. When an amount of azocasein, equal in protein concentration to that added as cytosolic protein in the complementation assay, was incubated with MF, the release of tyrosine was the same in both instances (Table 2.3). In the same kind of comparison, or when standard aliquots of the two were added together, the tyrosine release was again the same in these mixtures (Table 2.3). This indicates that the more-than-additive effect of combining MF and cytosol was due to possibility (c) above, i.e. that the insoluble myofibril-associated protease was capable of acting not only on its own particulate co-proteins, but also on soluble added substrates, viz. azocasein or cytosol.

2.3.1. Incubations at high ionic strength

There was a marked (4 - 5-fold) increase in the rates of tyrosine release when incubations were conducted in a buffer containing 0.6 M KCl rather than 0.1 M KCl (Table 2.3). The complementation observed at low ionic strength between MF and cytosol fractions was not evident at the high salt concentration; instead, there was much less proteolysis when the fractions were combined than when their individual

TABLE 2.2

PROTEASE ACTIVITIES IN SUBCELLULAR FRACTIONS OF RAT MUSCLE: ADDED AZOCASEIN

Constant amounts (i.e. 1 mg protein) of the various subcellular fractions were incubated in the presence of 2 mg azocasein. Protease activities were measured at low ionic strength, as described in Methods.

	ΔnMOLES TYROSINE/3 h
MF + azocasein	25
PMLS + azocasein	<0,1
ML + azocasein	<0,1
SR/SL + azocasein	<0,1
Cytosol + azocasein	<0,1

TABLE 2.3

EFFECTS OF AZOCASEIN AND CYTOSOL ADDITION ON PROTEASE ACTIVITIES IN MYOFIBRILS AT LOW AND HIGH IONIC STRENGTH

One milligram (protein) of MF fraction was incubated independently and with azocasein and/or cytosol in the various indicated amounts (at low and high ionic strength), protease activity being measured and expressed as described in Methods.

	PROTEOLYSIS (nmoles tyrosine/mg/3 h)	
	Low ionic strength	High ionic strength
MF	12	56
MF + Cytosol (2 mg)	26	10
MF + Cytosol (3 mg)	27,5	10
MF + Cytosol (5 mg)	28	9,5
MF + Azocasein (2 mg)	25	58
MF + Azocasein (3 mg)	29	59
MF + Azocasein (5 mg)	28	59
MF + Cytosol (1 mg) + Azocasein (1 mg)	26	20

activities were summed. Azocasein in the same concentration as cytosol protein produced a different result, in that the pattern of low salt complementation was reproduced; albeit to a less marked degree. Thus the cytosol contained an inh bitor not present in the azocasein solution.

Additions of 1% Triton X-100 or 1% sodium dodecyl sulphate to cytosol-containing incubations failed to remove the complementation effect with MF at low ionic strengths, suggesting that the inhibitor(s) was not membrane-associated. Furthermore, dialysis of the cytosol fractions (using dialysis tubing with a 12 000 molecular weight exclusion limit) failed to remove the inhibitor(s) revealed by incubations of MF with cytosol at high ionic strengths.

2.3.2. Time course

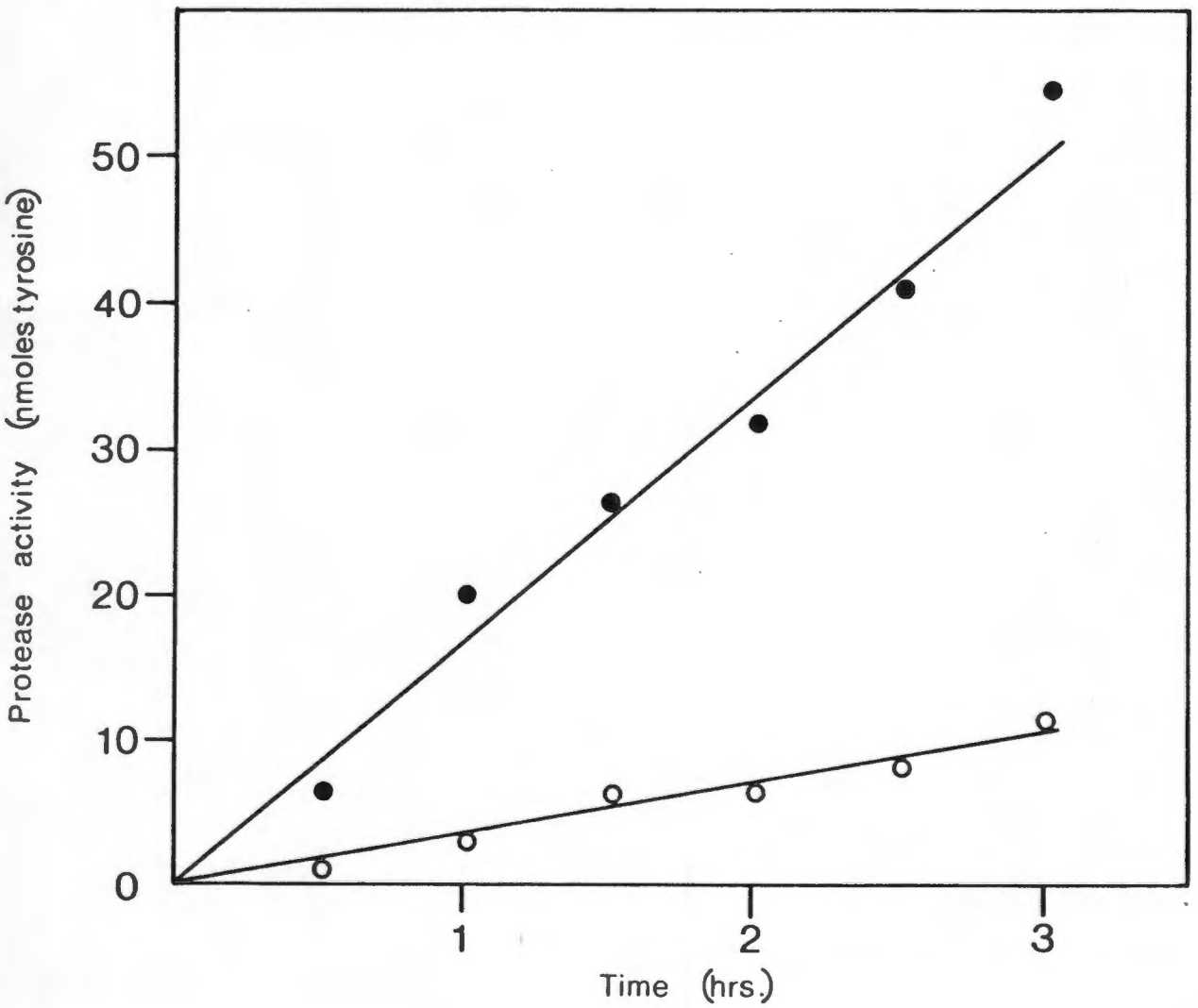
Rates of tyrosine released were linear with time over three hours in the MF fractions, at both low and high ionic strengths (Fig. 2.1).

2.3.3. Dose-response curve for myofibrils

The pattern of tyrosine released with increasing concentrations of MF fractions, incubated at low and high ionic strengths, was linear in both instances, although the activity at high ionic strength remained greater than that at low ionic strength at all concentrations of MF tested (Fig. 2.2).

Fig. 2.1

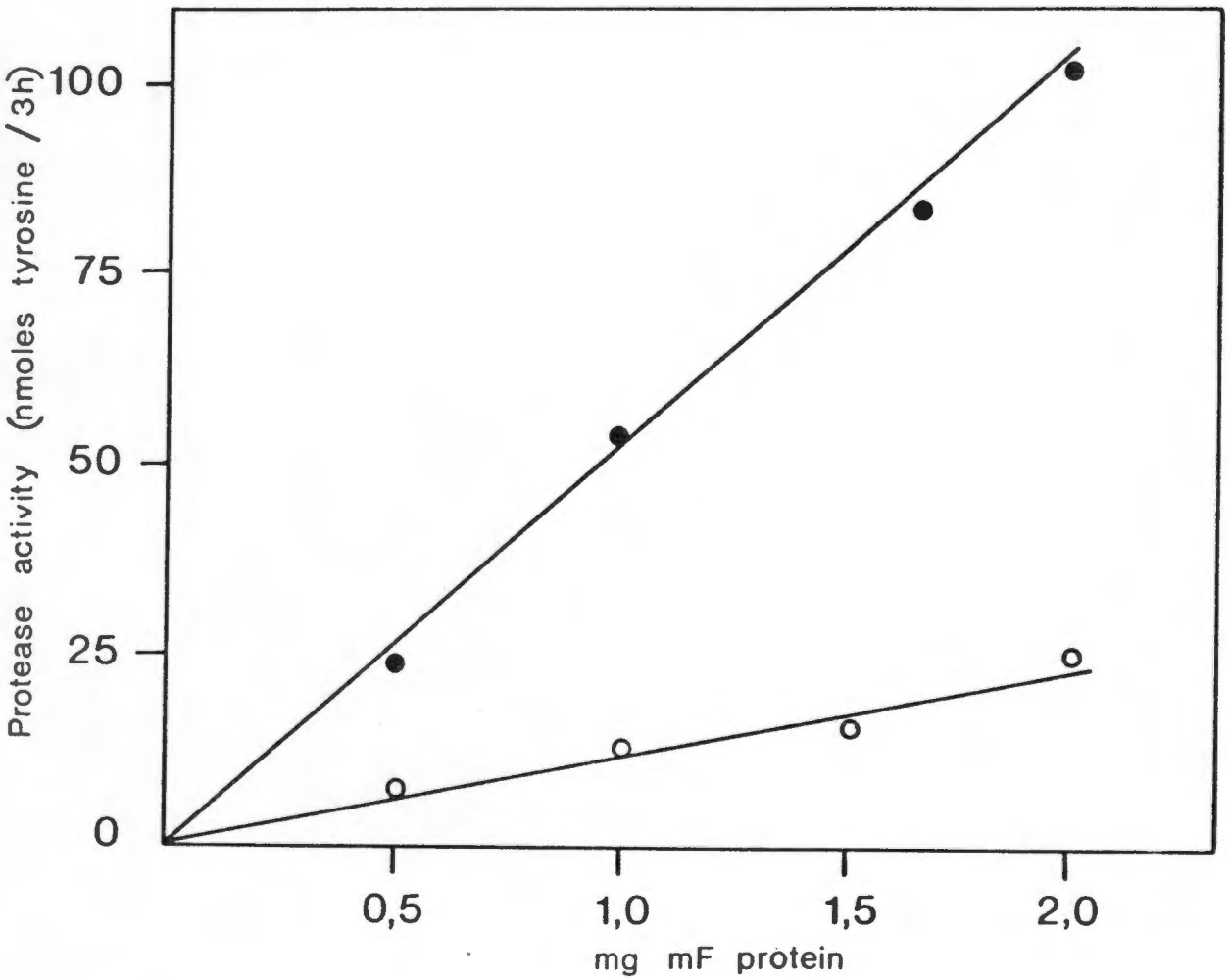
MEASUREMENT OF PROTEASE ACTIVITIES IN MYOFIBRILLAR FRACTIONS AT LOW AND HIGH IONIC STRENGTHS



Aliquots of MF protein (each containing 1 mg protein) were incubated at intervals up to 3 h, at low (o) and high (●) ionic strengths. Protease activities were measured as described in Methods.

Fig. 2.2

EFFECTS OF INCREASING AMOUNTS OF MYOFIBRILLAR FRACTIONS ON PROTEASE ACTIVITY AT LOW AND HIGH IONIC STRENGTHS



Increasing amounts (protein) of MF fractions were incubated at low (o — o) and high ionic strengths (● — ●). Protease activities were measured after 3 h, as described in Methods.

2.3.4. Azocasein concentrations

The addition of increasing amounts of azocasein to incubations containing MF and a constant concentration, at low ionic strength, revealed a gradual increase in the amount of tyrosine released until a plateau was reached, suggesting substrate saturation (Fig.2.3). This plateau value of tyrosine released was considerably greater than that achieved by MF alone at low ionic strengths, accounting for the similar degree of complementation observed when cytosol protein was added to MF. Under these conditions (see Fig. 2.3), the plateau activity of the low ionic strength system still fell short of the activity observed with MF alone at high ionic strengths, indicating that the filamentous form of the myofibrils was associated with diminished accessibility of the protease to susceptible protein substrates.

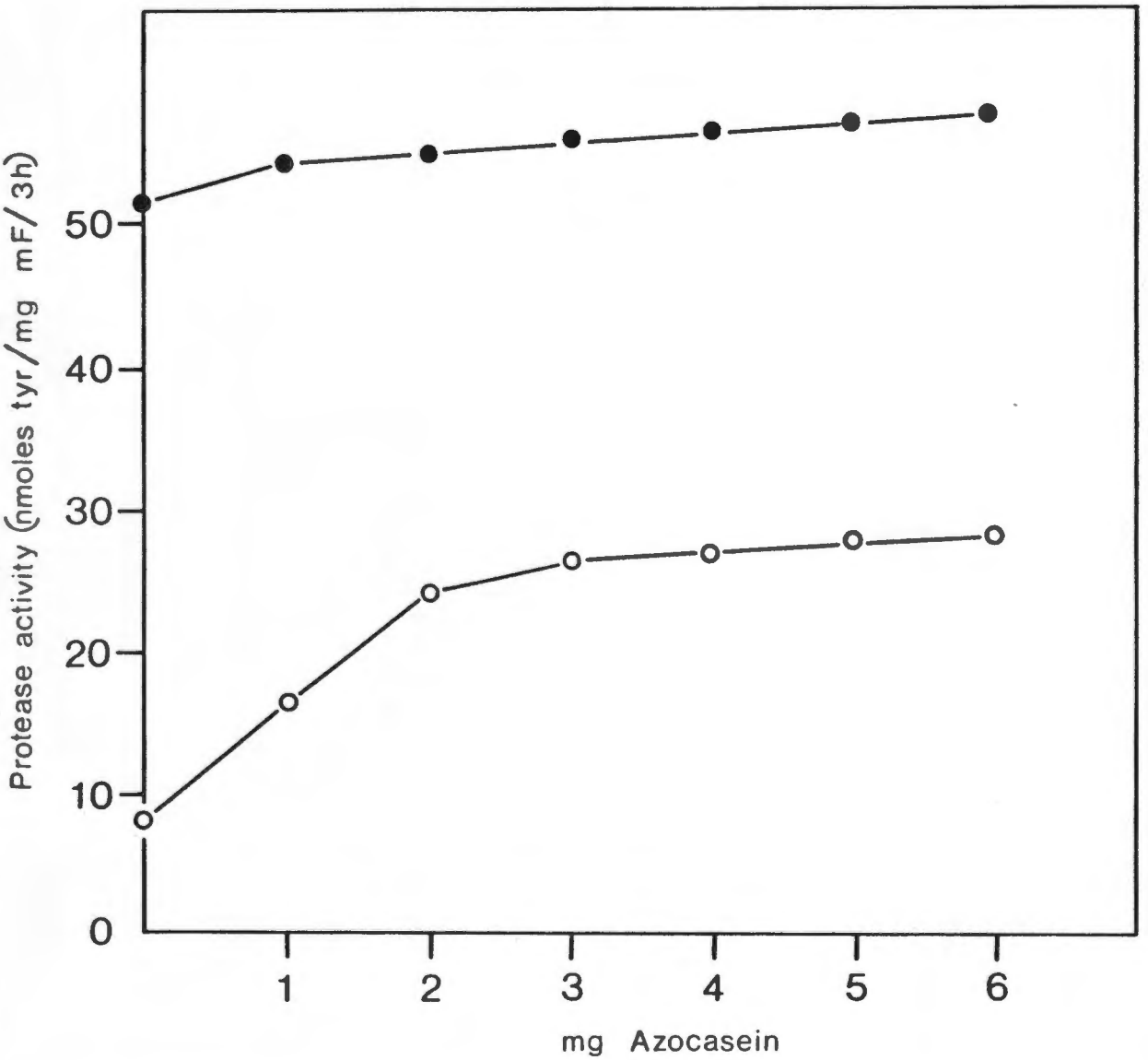
The level of tyrosine released from MF (0 azocasein) at high ionic strength was five-fold greater than that at low ionic strength and the addition of azocasein resulted in a small increment which rapidly reached a plateau as the azocasein was increased. This suggested that, at high ionic strength, more of the protease was available to act upon associated myofibrillar proteins so that upon addition of azocasein the marginal increase observed reflected near-saturation of the enzyme with susceptible substrates.

2.3.5. Effects of varying ionic strength on the protease

Chromatography on Sepharose 6B of the triton-washed myofibrils, after dissolution in 0.6 M KCl, resulted in elution

Fig. 2.3

EFFECTS OF AZOCASEIN ADDITION ON PROTEASE ACTIVITIES AT LOW AND HIGH IONIC STRENGTHS



Aliquots of MF fraction (each containing 1 mg protein) were incubated independently and in the presence of increasing amounts of azocasein, at low (o — o) and high (● — ●) ionic strengths. Protease activities were measured as described in Methods.

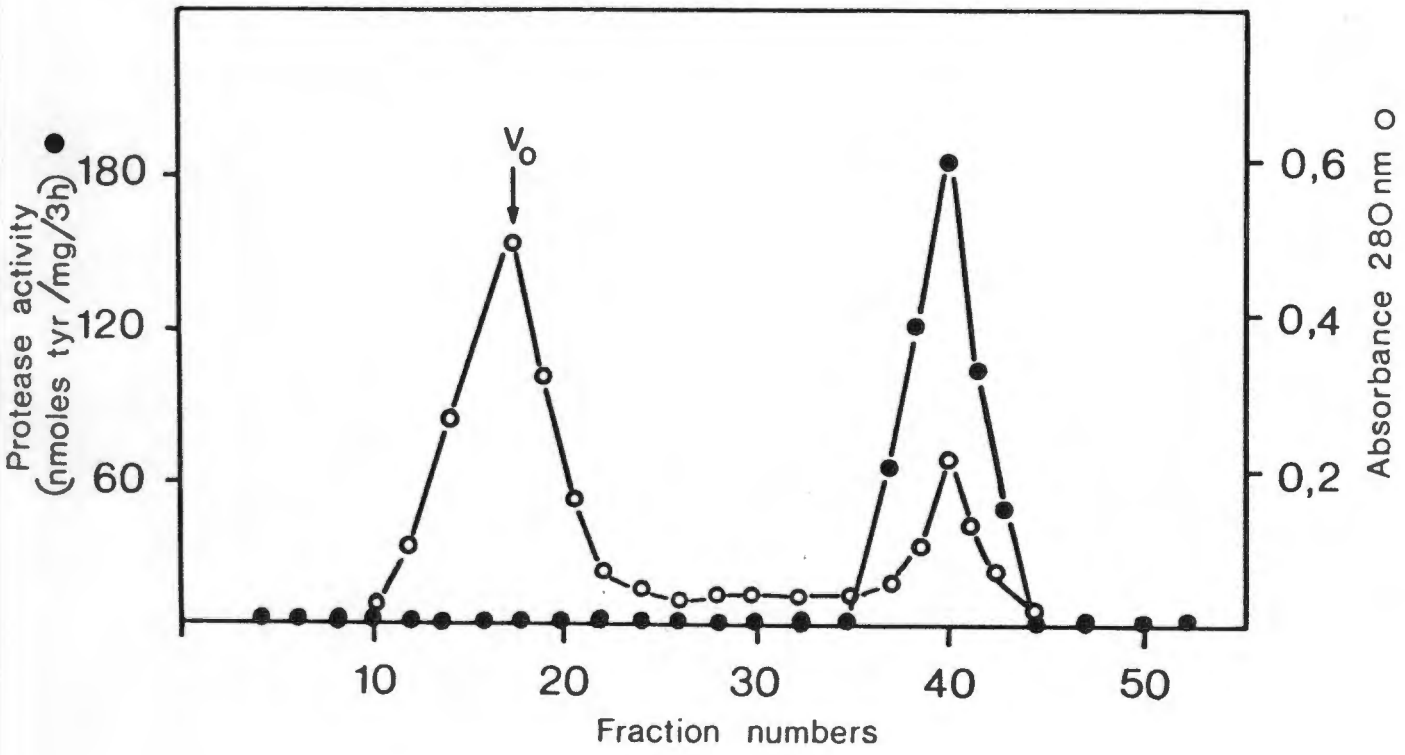
of chymase in the V_t region (Fig. 2.4). The protease in this form degraded azocasein and cytosol protein in a solution of low ionic strength (measured activity being 25 nmoles tyrosine/mg protease/3 h), but at high ionic strength the cytosol fraction was inhibitory (i.e. where the protease released 30 nmoles tyr/3 h with azocasein, only 8 nmoles tyr/3 h were released when the enzyme was incubated in the presence of equivalent cytosol protein). In addition, approximately 60% of the protease activity remained soluble when the column-purified form of the enzyme was dialysed against a low-salt buffer and centrifuged at 20 000 g. Thus the protease interacted with added cytosol protein in two ways, firstly, as protease acting on substrate at low salt concentrations, and secondly, as protease blocked by inhibitor(s) at high salt concentrations. When separated from actomyosin, the protease was largely soluble, but when subjected to low ionic strength conditions, a significant proportion of the activity behaved in an "actomyosin-like" manner and was accordingly precipitated.

2.3.6. Calcium-activated and myofibril-associated protease activities in various muscle types

Calcium-activated protease (CAP) activities were greater in the white compared with the red muscle fibre types, while the diaphragm (being a muscle containing almost equal proportions of white and red muscle fibres) had activity levels closer to that of white fibres (Table 2.4). On the other

Fig. 2.4

SEPHAROSE 6B CHROMATOGRAPHY OF MYOFIBRILLAR PROTEINS AT HIGH IONIC STRENGTH



Triton-washed myofibrils were solubilized in 0,6 M KCl, 1 mM DTT, 1 mM ATP, 50 mM tris-HCl (pH 7,4) and chromatographed on a Sepharose 6B column, previously equilibrated with the same buffer, all as described in Methods. One ml fractions were collected, absorbances being read at 280 nm and protease activities determined as described in Methods.

TABLE 2.4

CALCIUM-ACTIVATED AND MYOFIBRIL-ASSOCIATED PROTEASE ACTIVITIES IN VARIOUS RAT MUSCLE TYPES

Constant amounts (protein) of MF fraction and PMLS fractions (following 'pH 5 step'), prepared from the various rat muscles as described in Methods, were subjected to assays of their respective myofibril-associated and calcium-activated protease activities, as described in Methods. Similar results were obtained in three separate experiments.

MUSCLE TYPE	CALCIUM-ACTIVATED PROTEASE ACTIVITY (nmoles tyrosine/mg/3 h)	MYOFIBRIL-ASSOCIATED PROTEASE ACTIVITY (nmoles tyrosine/mg/3 h)
Diaphragm (red + white)	8	57
Soleus (red)	1	49
Heart	2	35
Psoas (white)	14	17
EDL (white)	10	18
Gluteus medius (white)	9	11

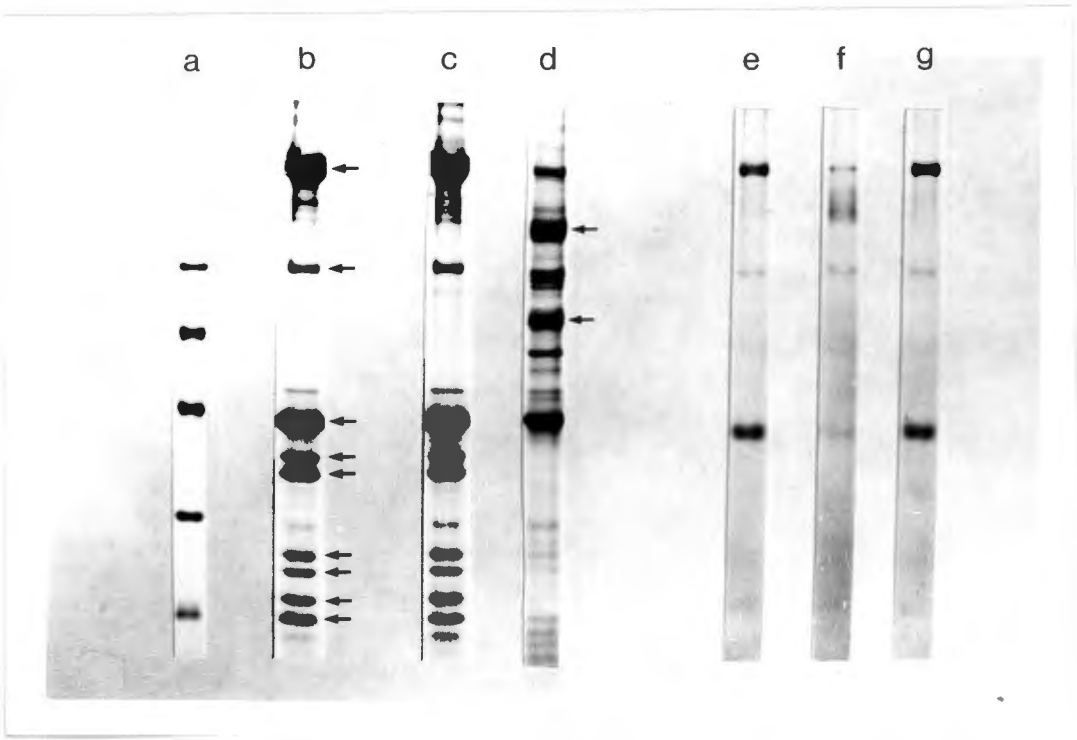
hand, myofibril-associated protease activity was clearly greater in muscles containing red rather than white muscle fibres, although the diaphragm once more was noted to have high activity levels of this protease in muscle homogenates. Cardiac muscle contained a low order of CAP activity and a considerable level of the myofibril-associated protease.

2.3.7. Degradation of myofibrillar proteins

Despite the tyrosine release observed in the case of MF autolysis at low ionic strength, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the MF pellet before and after incubation revealed no detectable change in the staining pattern of myofibrillar proteins. However, SDS-PAGE of the MF fraction incubated at high salt concentrations revealed extensive removal of myosin heavy chains with the formation predominantly of heavy meromyosin (HMM) - and light meromyosin (LMM) - like products (Fig. 2.5). Tropomyosin and myosin light chains were also readily degraded while actin removal was less marked. The presence of azocasein did not alter this degradation pattern, but that of cytosol markedly retarded the removal of myofibril-associated heavy chains under high salt conditions (Fig. 2.5). These results point to the presence in the cytosol of an endogenous inhibitor(s) which operates effectively only when its target protease is soluble. Alternatively, there is the less likely possibility that the inhibitor is "activated" at high salt concentrations.

Fig. 2.5

SDS-POLYACRYLAMIDE GEL ELECTROPHORETIC ANALYSIS OF MYOFIBRILLAR PROTEINS, BEFORE AND AFTER PROTEOLYSIS



Analysis by SDS-polyacrylamide gel electrophoresis of a myofibrillar (MF) pellet prepared from mixtures incubated at low and high ionic strengths, in the absence and presence of added cytosol or azocasein, all as described in Methods. (a) = molecular weight standard; (b) + (e) = MF standards; (c) = MF 3h, 37°C, low ionic strength; (d) = MF 3h, 37°C, high ionic strength; (f) = MF + azocasein, 3h, 37°C, high ionic strength; (g) = MF + cytosol, 3h, 37°C, high ionic strength.

Molecular weight standards in order of decreasing size: 94 000; 67 000; 43 000; 30 000 and 20 000. Arrows in lane (b), from above downwards, represent: myosin heavy chain; α-actinin; actin; troponin-T; tropomyosin; myosin light chain 1; troponin-I; myosin light chain 2; troponin-C. Arrows in lane (d) represent heavy meromyosin-like fragment (above) and light meromyosin-like fragment (below).

2.3.8. Properties of the myofibril-associated protease

The pH optimum of the MF protease was 8.5, as found by numerous other authors (see Introduction) (Fig. 2.6).

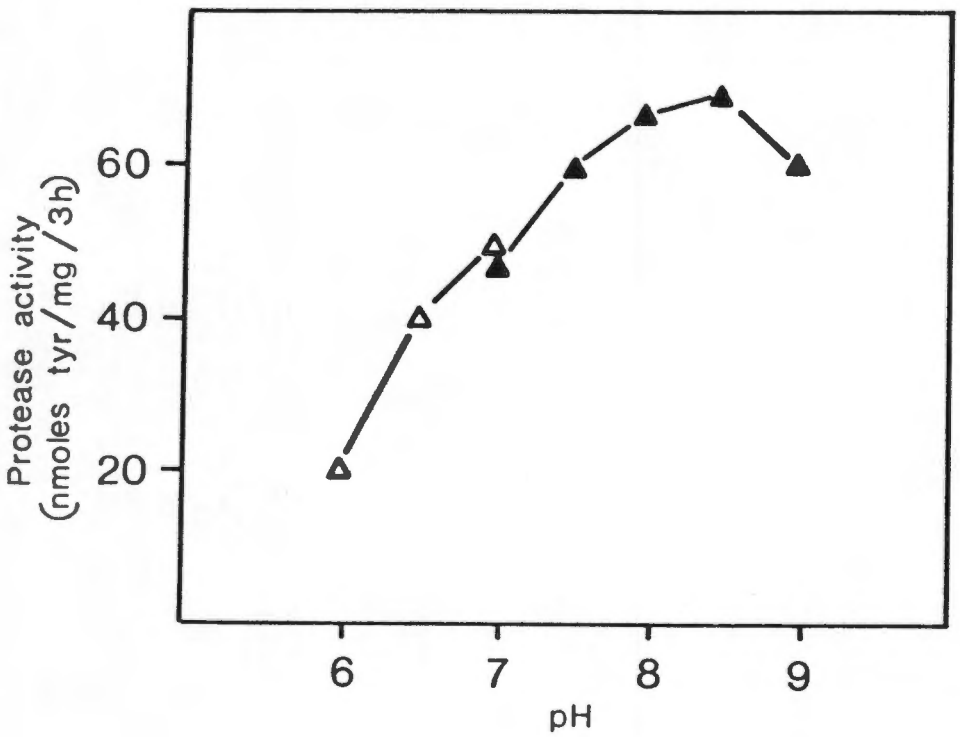
2.3.9. Substrate specificity

Casin and azocasein were both good substrates for MF-protease, with tyrosine measurement by fluorimetry providing a more sensitive assay than the colorimetrically assessed release of azo groups in the latter case.

[^{14}C methyl]globin and [^{14}C-NEM]-MF were degraded to give radioactive TCA-soluble fragments when incubated with MF fractions derived from skeletal muscle homogenates (Table 2.5). Protease activity measured in this way also followed the pattern of ionic strength enhancement and chymostatin inhibition revealed by the previously described assay methods. However, the use of radio-labelled protein substrates was limited in the reconstitution studies in that observed decreases in the amounts of TCA-soluble products formed upon combination of various subcellular fractions reflected either the presence of protein inhibitor(s) or similarly the dilution of radioactive substrate by competing cold protein substrate(s). It was therefore decided to determine protease activities routinely by measuring tyrosine fluorimetrically; this was in any case a sensitive and highly reproducible assay. Moreover, as tyrosine is present in most proteins at a reasonably constant level (Fulks et al, 1975), competing "added" protein substrates did not dilute out the

Fig. 2.6

pH OPTIMUM OF PROTEASE ACTIVITY



An MF fraction (1 mg protein) in high salt (0,5 M KCl) was incubated in 50 mM imidazole buffer (pH △ 6-7) or 50 mM tris-HCl buffer (pH ▲ 7-9); protease activities were expressed and measured as described in Methods.

TABLE 2.5

PROTEASE ACTIVITIES WITH LABELLED PROTEIN SUBSTRATES

One milligram (protein) amounts of MF fraction were incubated in the presence of [^{14}C methyl] globin or [^{14}C NEM] myofibril substrate (at low and high ionic strength), in the absence and presence of protease inhibitors. Protease activities were measured as expressed in Methods.

	ACTIVITY WITH [^{14}C METHYL]GLOBIN SUBSTRATE (dpm/h)	ACTIVITY WITH [^{14}C NEM]MYOFIBRIL SUBSTRATE (dpm/h)
MF (low i)	1300	1050
MF (high i)	3000	2400
MF + Chymostatin (high i) (20 µg/ml)	420	300
MF + Leupeptin (high i) (20 µg/ml)	2700	2200
MF + Pepstatin (high i) (20 µg/ml)	2800	2450

product but simply added to it. The presence of inhibitor(s), on the other hand, was associated with a drop in the measured tyrosine.

2.3.10. Thermostability (+ ATP)

In view of the observations made in the case of certain proteases concerning thermostability in the presence of ATP or citrate (Rose et al, 1979), the ability of the MF protease to withstand heating at various temperatures, in the presence and absence of these agents was tested; activity was lost rapidly in every case.

2.3.11. Inhibitors and possible activators

Amongst the antibiotic inhibitors tested, chymostatin was strongly inhibitory, whilst leupeptin and pepstatin had no effect even at dose levels of 40 µg/ml (Table 2.6). Both the serine protease inhibitors phenylmethyl sulphonyl fluoride, and di-isopropylphosphofluoridate were inhibitory at concentrations of 1 mM (Table 2.6).

Addition of several agents including Ca^{++} (1 mM), ATP (2 mM), triton X-100 (1%) and DTT (1 mM) caused no change in the protease activity. A KCl concentration curve revealed maximal activity at 0,6 M (data not shown).

2.3.12. Treatment of rats with Compound 48/80

The muscles of rats subjected to the 5-day programme of mast cell degranulation induced by compound 48/80, contained between

TABLE 2.6

EFFECTS OF INHIBITORS ON MF PROTEASE

An MF fraction (1 mg per assay) was incubated in the absence and presence of known protease inhibitors at various concentrations. Protease activities were determined thereafter as described in Methods.

INHIBITOR		% INHIBITION
Chymostatin	8 µg/ml	85
	10 µg/ml	90
	20 µg/ml	90
PMSF	1 mM	80
(Phenylmethyl sulphonyl fluoride)	5 mM	90
DFP	1 mM	90
(Diisopropylphospho-fluoridate)	5 mM	90
NEM	1 mM	70
(N-ethylmaleimide)	5 mM	75
Leupeptin	20 µg/ml	0
	40 µg/ml	0
Pepstatin	20 µg/ml	0
	40 µg/ml	0
Iodoacetate	1 mM	0
	5 mM	0

5-10% of the MF protease activity present in untreated control tissue, independent of the conditions of the assay (Table 2.7). SDS-PAGE analysis of the myofibrils revealed no degradation of myosin heavy chains in these incubations (Fig. 2.7).

Histological examination using Giemsa and Bismarck Brown stains of skeletal muscle samples from the treated rats confirmed that mast cell degranulation was virtually complete (Fig. 2.8).

2.3.13. Cell cultures

In view of the cellular heterogeneity of intact skeletal muscles, the L8 line of muscle cells (gift of Prof. D. Yaffe, Weizmann Institute (Israel)) was examined in order to establish the cellular source of myofibril-associated protease(s). No 'MF protease' activity was detectable (beyond the limit of the sensitivity of the assay) in fractions prepared from these post-fusion cells (Table 2.5). SDS-PAGE analysis of the MF fraction confirmed myosin and actin to be the dominant protein components of the incubated material, and this did not show evidence of degradation following incubation at low or high salt concentrations (Fig. 2.7).

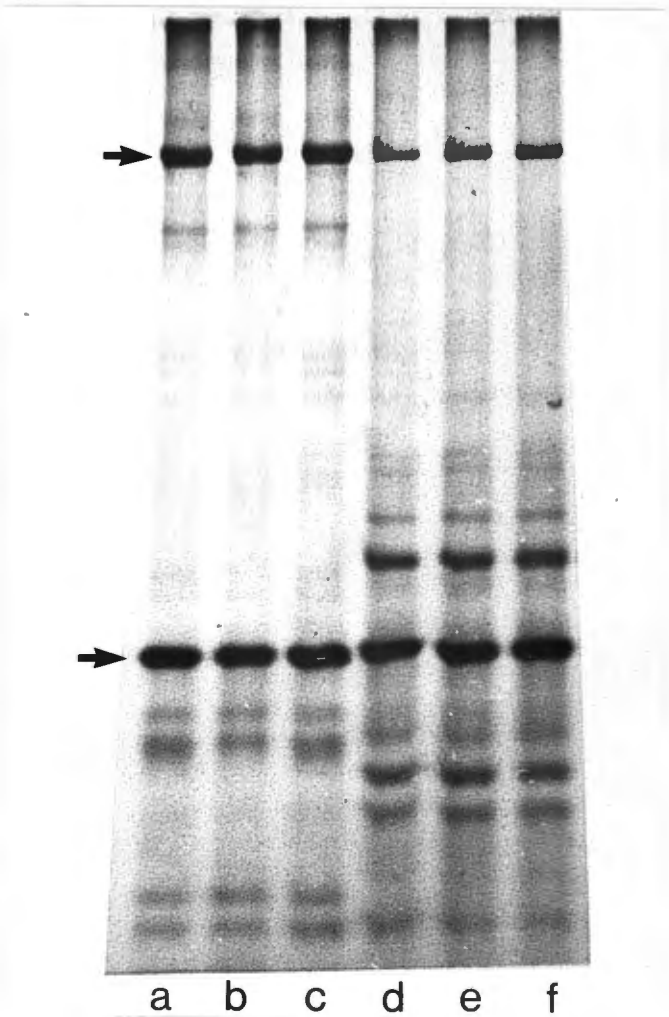
TABLE 2.7

MF fractions from skeletal cell culture extracts and rat diaphragm homogenates derived from control and compound 48/80 treated rats were prepared as described in Methods. Protease activities were measured in the absence and presence of 2 mg casein, at low and high ionic strength, as described in Methods.

	PROTEOLYSIS (nmoles tyrosine/mg/3 h)	
	Low ionic strength	High ionic strength
Rat skeletal muscle cells in culture:		
MF	0,1	0,1
MF + Casein	0,1	0,1
Rat skeletal muscle		
MF	12	56
MF + Casein	28	64
Compound 48/80 - treated rat muscle		
MF	1,5	2
MF + Casein	1	2

Fig. 2.7

MYOFIBRIL PROTEIN DEGRADATION (SKELETAL CELL CULTURE EXTRACTS AND 48/80-TREATED RAT DIAPHRAGM HOMOGENATES); ANALYSIS BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS



Myofibrillar (MF) fractions prepared from skeletal cell culture extracts and 48/80-treated rat diaphragm homogenates were incubated at low and high ionic strengths (as described in Methods) and thereafter subjected to SDS-polyacrylamide gel electrophoresis.

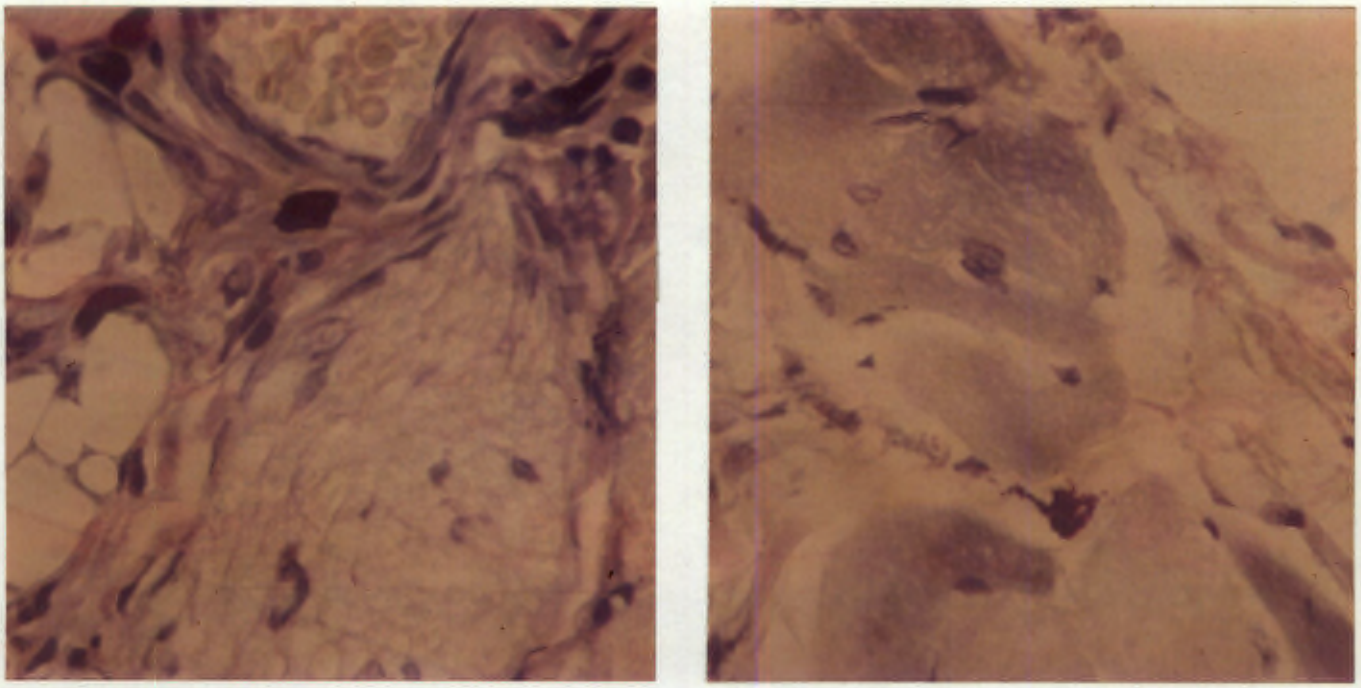
MF from 48/80-treated rat diaphragm: (a) = MFO'; (b) = MF 3h, 37°C, low ionic strength; (c) = MF 3h, 37°C, high ionic strength.

MF from skeletal muscle cells: (d) = MFO'; (e) = MF 3h, 37°C, low ionic strength; (f) = MF 3h, 37°C high ionic strength.

Arrows: Above = myosin heavy chain; below = actin.

Fig. 2.8

MAST CELL DEGRANULATION



Rat skeletal muscle (i.e. diaphragm) obtained from normal rats (left) and 48/80-treated rats (right) - as described in Methods - examined histologically by Giemsa stain. The mast cells appear deep blue because of staining of granules. Slide prepared and photographed by Professor A.G. Rose.

2.4. DISCUSSION

In confirmation of the work of other authors (see Introduction) it has been ascertained that rat skeletal muscle contains an alkaline protease activity which is detectable in the particulate fraction of homogenates prepared at low salt concentrations. The novel findings in this study are three-fold:

First, that at low ionic strength, the enzyme is capable of degrading native myofibrillar proteins at a relatively slow rate, while it also acts on added soluble (cytosolic) protein substrates, despite the apparent presence in the latter of a proteinaceous inhibitor;

Second, that when the myofibrils are dissolved at high salt concentrations, the alkaline protease enters into solution and is optimally active, degrading the myofilaments rapidly to free amino acids (e.g. tyrosine) as well as to intermediate HMM- and LMM-like fragments. Upon addition of the cytosol fraction to this high salt system, however, there is marked retardation (inhibition) of the degradative process, because the cytosolic inhibitor can apparently interact well with the solubilized enzyme under these conditions;

Third, that the protease is independently liable to precipitate at low ionic strength, and associates with actomyosin in muscle homogenates because of this property. It is because of this strong association that the term 'myofibrillar alkaline protease' (MAP) has been used in the past (Griffin and Wildenthal, 1978).

Clues to the true cellular origin of the major protease of fresh muscle homogenates became available when certain properties (immunologic, physical and biochemical) were found to be shared by this protease and the peritoneal mast cell-derived, chymotrypsin-like serine protease called chymase (Woodbury et al, 1978). Mast cell degranulation leads to the loss of most of this alkaline protease activity (McKee et al, 1979; Edmunds and Pennington, 1981), and this has been confirmed in this study.

Chymase activities have previously been noted to be higher in red than in white muscle fibre types (Katunuma and Kominami, 1977), the higher level of the alkaline protease presumably reflecting a greater population of mast cells in red muscle.
These trends have been confirmed in the present work in respect of different muscle types, although calcium-activated protease activities showed the reverse pattern viz. a predominance in white muscle fibre types.

These results therefore present confirmatory as well as new evidence in favour of an exclusive non-muscle cell (viz. mast cell) location of the alkaline serine protease of muscle homogenates - and would suggest that this enzyme and the mast cell chymase are one and the same. This would accord it relatively little importance in the study of intracellular muscle cell protein turnover. Nevertheless, of interest is the fact that this protease can degrade myofibrillar as well as cytosolic native proteins. The enhancement of this

activity at high ionic strength, together with its inhibition under such conditions by endogenous cytosolic inhibitor(s) provides a model for the study of protease-substrate-inhibitor interactions of other cellular proteases in this manner, especially the two-phase features of this system. The mast cell is distributed throughout various tissues of the body (Riley, 1959) and contains granules in which histamine, chymase, heparin and acid hydrolases are packaged (see Pepys and Edwards, 1979). The functions of mast cells in terms of physiology and pathophysiology at the cellular and molecular levels remain to be clarified.

CHAPTER 3

A HIGH MOLECULAR WEIGHT CYSTEINE ENDOPEPTIDASE FROM RAT SKELETAL MUSCLE

3.1. INTRODUCTION

Current models for intracellular protein degradation pathways in skeletal muscle propose initial, selective proteolytic "attacks" which take place outside the lysosomes and which are rate-limiting for the overall degradation to the level of amino acids (Kominani et al, 1975; Katunuma et al, 1977). Several non-lysosomal proteases (soluble and particulate), active in the neutral to mildly alkaline pH range, have been identified in skeletal muscle and other tissues. These include the Ca^{++}-activated thiol protease which may occur in two forms with differing Ca^{++} sensitivity (Reville et al, 1976; Dayton et al, 1981); a serine protease that hydrolyses muscle proteins (Koszalka and Miller, 1960), a thiol protease that degrades insulin (Duckworth et al, 1972) and a particulate, "myofibril-associated" protease with an alkaline pH optimum (Mayer et al, 1974; Yasogawa et al, 1978; Noguchi and Kandatsu, 1970). Data from several laboratories (Paston and Almqvist, 1966; McKee et al, 1979; Libby and Goldberg, 1980; Edmunds and Pennington, 1981) indicate firstly that the "alkaline" serine protease activity of muscle and other tissue homogenates, resides predominantly or wholly in mast cells, and secondly that ridding the tissue of this activity by means of prior mast cell degranulation does not influence significantly the overall rate of protein degradation in intact muscles (Libby and Goldberg, 1980) or perfused hearts (McKee et al, 1979).

The aim was to identify enzymes which are definitely localized

within muscle cells (rather than in mast cells) and which have properties compatible with substrate selectivity and physiological regulation known to occur in circumstances such as denervation, starvation, diabetes and hyperthyroidism Recent reports of an ATP-dependent, multi-component pathway of selective protein degradation in reticulocytes (Ciechanover et al, 1978; Hershko et al, 1979, 1980, 1981), which implicate the heat-stable small protein, ubiquitin as a re-usable cofactor for degradation, have stimulated one to examine rat skeletal muscles as a source of elements of such a system. The properties of an ATP-dependent liver enzyme have already been reported (Rose et al, 1979; De Martino and Goldberg, 1979).

The only enzyme of high molecular weight (340000) which has been fully described in detail in skeletal muscle is the zinc-sensitive aminoendopeptidase (Hydrolase H) isolated from rabbit tissue by Okitani et al (1981); this enzyme does not apparently interact with ATP. Two brief reports of successful extractions, from human and rat muscles, of high molecular weight proteinases, possibly related to the reticulocyte and liver enzymes mentioned above, have recently appeared; one of these activities was inhibited by ATP (Hardy et al, 1981), while the other was activated by ATP (Etlinger et al, 1981), in a manner apparently involving a heat-stable "APF-1" fraction similar to ubiquitin (Ciechanover et al, 1980[b]).

In the course of these studies, the problem of "contamination" of skeletal muscle homogenates (prepared from animals not treated with compound 48/80) by the mast cell protease (chymase) was overcome by preparation of extracts at low ionic strength when chymase coprecipitates quantitatively with the myofibrillar fraction sedimented at low speed. Supernatant preparation obtained in this way contained no detectable chymostatin-inhibitable protease activity (i.e. chymase activity) and this fraction, freed of particles and membranes by high-speed centrifugation, was then used as a source of muscle cell-specific proteases and other components.

A soluble cysteineendopeptidase of high molecular weight demonstrably different from hydrolase H characterized as an amino endopeptidase in rabbit muscle (Okitani et al, 1981), was identified and purified by an apparent factor of 1000, using column chromatographic procedures. Ubiquitin (APF_1: Ciechanover et al, 1980a) was also prepared from rat muscle extracts and from rat or human erythrocytes, but no ATP-dependent complementation on the lines of the reticulocyte system of Hershko and co-workers (Ciechanover et al, 1978; Hershko et al, 1979) could be detected. A study of chicken reticulocytes also failed to reveal ATP-dependent complementation, although a high molecular weight protease, similar to that of the rabbit reticulocyte system (Hershko et al, 1979) and to that described here, was detected. Nevertheless, the enzyme described here may be part of an ATP-

dependent, multi-component proteolytic system similar to that already known to be present in reticulocytes.

3.2. MATERIALS AND METHODS

3.2.1. Labelled protein substrates

[^{14}C methyl]-globin (2.5 x 10^6 dpm/mg) was prepared by labelling bovine haemoglobin according to the method of Rice and Means (1971), followed by haem extraction as described by Schapira et al (1968). In addition, [^{14}C-N-ethyl-maleimide]-labelled myofibrillar proteins (for preparation, see Chapter 2) and [I^{125}]labelled myofibrillar proteins (prepared as described by Ciechanover et al, 1980) were additional radio-labelled substrates, tested for their susceptibilities.

3.2.2. Fractionation of skeletal muscle extracts

Long-Evans hooded rats (250 - 350 grams) of both sexes, inbred in this laboratory and maintained on standard rat chow, were anaesthetized with intraperitoneal injections of pentobarbitone and killed by decapitation. Muscles from both hind limbs were rapidly dissected out, freed of fat and connective tissue, weighed, and cut into fine pieces chilled in ice. A homogenate (1:4, w/v, in 20 mM sodium phosphate buffer, pH 7.1, also containing 0.5 mM DTT), was prepared from the tissue fragments with an Ultraturrax homogenizer, operated at setting 6, using 2 x 30 second bursts and with continuous cooling in ice.

The homogenate was initially centrifuged in the cold at 800 g for 15 minutes, and the resulting supernatant centrifuged twice further, first at 20 000 g for 15 minutes, and then at 100 000 g for 60 minutes in a Beckman SW 36 rotor. This left a particle- and membrane-free, high-speed supernatant fraction (HSSN), which was dialysed for 18-24 hrs against 250 volumes of the ice-cold homogenized buffer solution. The dialysed HSSN was then kept at 70°C for 10 minutes after the addition of ATP (final concentration 2 mM).

Denatured protein was pelleted by centrifugation at 20 000 g for 20 minutes. The supernatant fraction (42 ml) was applied to a DEAE-cellulose (Whatman DE 52) column (1,4 x 20 cm), previously equilibrated with the homogenization buffer. The adsorbed protein was eluted with a linear KCl gradient (0 - 0,5 M), and fractions (2 ml) were collected for assays of protease activity (see below), and absorbance measurements at 280 nm.

Sepharose 6B (or 4B) columns (60 x 0.9 cm) were equilibrated in the cold with 50 mM Tris-HCl and 0.5 mM DTT, pH 7.5, before the application of various samples at 1-5% of bed volumes. Fractions of 1 ml were collected at a flow rate of 2 ml/h. The columns were calibrated with known marker proteins.

3.2.3. Protease assays

Protease activity was routinely measured by the hydrolysis of [^{14}C methyl]-globin. Each assay contained 100 mM Tris-HCl, 5 mM $MgCl_2$, 0.5 mM DTT, pH 7.5, together with the radioactive substrate (1.5×10^4 dpm), all in a final volume at 250 µl. Incubations were performed at 37°C for 60 min, after which 200 µl 10% ice-cold trichloroacetic acid (TCA) containing 5 mg/ml carrier bovine serum albumin was added to each to stop the reactions. Aliquots (200 µl) of the supernatant fractions obtained by centrifugation were mixed with 5 ml Instagel (Packard Instrument Co., Downer's Grove, Illinois, USA) and counted in a Beckman LS 9000 LS Scintillation Counter. Protease activity was expressed as dpm released in acid-soluble form per h, after subtraction of values for control reactions not containing added enzyme. Acid-soluble products of proteolysis were examined by chromatography of incubated assay mixtures on a Sephadex G25 (30 x 0.9 cm) column, followed by radioactivity analysis as described above.

Assay conditions with [^{125}I]labelled myofibrils or [^{14}C NEM] labelled myofibrils were similar, with the exception that assay mixtures contained (4×10^5) dpm or (1.5×10^4) dpm per test-tube, respectively, when these substrates were tested. The rates of enzymatic hydrolysis of L-leucine-2-naphthylamide (Leu-N-Nap) and α-N-benzoyl-L-arginine 2-naphthylamide (Bz-Arg-N-Nap) were measured by a modified method of Hardy and Pennington (1979). With Leu-N-Nap as

substrate, each assay mixture consisted of 50 mM Tris-HCl, 1 mM EDTA, 0.5 mM substrate and sample in a final volume of 0.5 ml at pH 7.5. After incubation for 1 hr at 37°C, 2 ml of ice-cold ethanol was added to each tube and the liberated naphthylamide measured fluorimetrically (excitation 339 nm, emission 403 nm). A standard curve using pure naphthylamine was obtainedand the enzyme activity accordingly expressed as nmoles naphthylamide released/min. In the case of assay mixtures containing Bz-Arg-N-Nap as substrate, the same procedure was followed but in these instances the substrate concentration was 2 mM.

Protein determinations were performed by the method of Lowry et al (1951), as modified by Hartree (1972), with bovine serum albumin as standard.

3.2.4. Gel electrophoresis

An aliquot of the purified enzyme from the Sepharose 6B column was concentrated by TCA precipitation and subjected to SDS-polyacrylamide gel electrophoresis according to the method of Porzio and Pearson (1977).

3.2.5. Chicken reticulocytes

Reticulocytosis (80 - 90% of total cell count) was induced in chickens by the injection of phenylhydrazine as described by Etlinger and Goldberg (1977).

3.2.6. Reticulocyte lysate

Cells obtained by centrifugation of heparinized blood at 2000 rpm were washed twice in ice-cold saline and incubated (37°C, 2 h) with 0.2 mM 2,4-dinitrophenol and 20 mM deoxyglucose, to deplete cellular ATP (Hershko et al, 1978). Following additional washings, the cells were lysed with 1,6 volumes of deionized water containing 1 mM DTT, and particulate material removed by centrifugation at 80 000 g for 90 minutes.

3.2.7. Fractionation on DEAE-cellulose

All operations were at 0-4°C. Thirty ml lysate was applied to a 1.5 x 12 cm column of DEAE cellulose (Whatman DE-52) equilibrated with 20 mM sodium phosphate (pH 7.1) containing 0.5 mM DTT. Adsorbed proteins were eluted with 50 ml of the above buffer containing 1 mM DTT. The last two-thirds of this material containing haemoglobin were collected and designated fraction I (ubiquitin) (Ciechanover et al, 1978).

The remaining protein was then eluted with 70 ml of a solution containing 10 mM Tris-HCl, 0.5 mM DTT, 0.5 M KCl (pH 7.1). Solid ammonium sulphate was added to 90% saturation (65 g per 100 ml of solution), mixed well, and the suspension kept in ice for 30 minutes. Precipitated protein was collected by centrifugation and taken up in 2 ml of a solution containing 50 mM Tris-HCl, 0.5 mM DTT (pH 7.5). This material was called fraction II. One ml of this fraction was applied to a Sepharose 4B column (60 x 0.9 cm)

and 1 ml fractions were collected at a flow rate of 2 ml/h. Protease activities of the various preparations and fractions were determined using [^{14}C methyl]globin as substrate - assay conditions and procedures being the same as for the skeletal muscle preparations described above. Protease activities of the various fractions independently and upon reconstitution were determined in the presence and absence of 5 mM ATP.

3.2.8. Materials

[^{14}C-methyl]-formaldehyde (16.7 mCi/nmol) was obtained from Amersham, England. Chymostatin, leupeptin and pepstatin were purchased from the Peptide Institute, Protein Research Founcation, Japan.

DTT, ATP, creatine kinase, creatine phosphate, compound 48/80, L-tri-iodothyronine, L-Leu-N-Nap, Bz-Arg-N-Nap and naphthylamine were obtained from Sigma Chemical Company, USA, and all other chemicals used were Analytical Reagent grade.

3.3. RESULTS

3.3.1. Purification of protease

The heat-treated, high-speed supernatant (HSSN) contained little protease activity in the presence or absence of ATP (Table 3.1). Dialysis of this fraction (dialysis tubing with a 3 500 molecular inclusion limit) caused a 10-fold increase in activity. This suggested that dialysable endogenous inhibitors were present in the supernatant; such inhibition could also have been "apparent" (removal of competing peptide substrate(s)). No inhibitor could be detected, however, in a reconstitution experiment using an undialysed HSSN preparation (see below). As zinc chloride was subsequently shown markedly to inhibit the purified enzyme (Table 3.2), the effect of a zinc ion chelator (imidazole) on the undialysed HSSN fraction was studied. This failed to enhance the tissue activity (results not shown), suggesting that the removal of zinc ions was not the explanation for enhancement following dialysis. After the heating step, there was a further increase in specific activity of the extract, with an extensive loss of total protein (Table 3.1).

When the dialysed and heat-treated fraction was applied to an anion exchange column (DEAE-cellulose), all the red-pigmented protein (presumably residual myoglobin) was washed off the column; this contained no protease activity. A virtually colourless band of protein remained adsorbed onto the column. This was eluted during the application of a

TABLE 3.1

PURIFICATION OF HIGH MOLECULAR WEIGHT ENDOPROTEASE

All procedures and assays were carried out as described in Methods using 8 g of rat hindleg muscle as starting material.

Fraction	Protein (mg)	Total Activity (dpm)	Specific Activity (dpm/mg)	Apparent-fold Purification	Yield
High-speed Supernatant	350	19500	56	-	-
Dialysed high-speed supernatant	340	200000	588	x 1	-
Heat-treated material	106	325000	3066	x 5	100%
Activity peak on DEAE-cellulose	1,6	320000	200000	x 340	98%
Activity peak on Sepharose 6B	0,5	308000	616000	x 1048	95%

TABLE 3.2

EFFECTS ON ENDOPROTEASE OF VARIOUS INHIBITORS AND ACTIVATORS

An enzyme fraction purified as described in Fig. 2, was incubated at 37°C for 1 h in the presence and absence of various inhibitors and/or activators. The assays with radioactive globin were carried out as described in Methods.

Agent		% Inhibition
$ZnCl_2$	(0,1 mM)	80
Parachloromercuri-sulphonylfluoride	(0,1 mM)	70
N-ethylmaleimide	(1 mM)	60
Iodoacetate	(5 mM)	0
Chymostatin	(40 µg/ml)	5
Leupeptin	(40 µg/ml)	0
Pepstatin	(40 µg/ml)	0
EDTA	(5 mM)	0
EGTA	(5 mM)	0

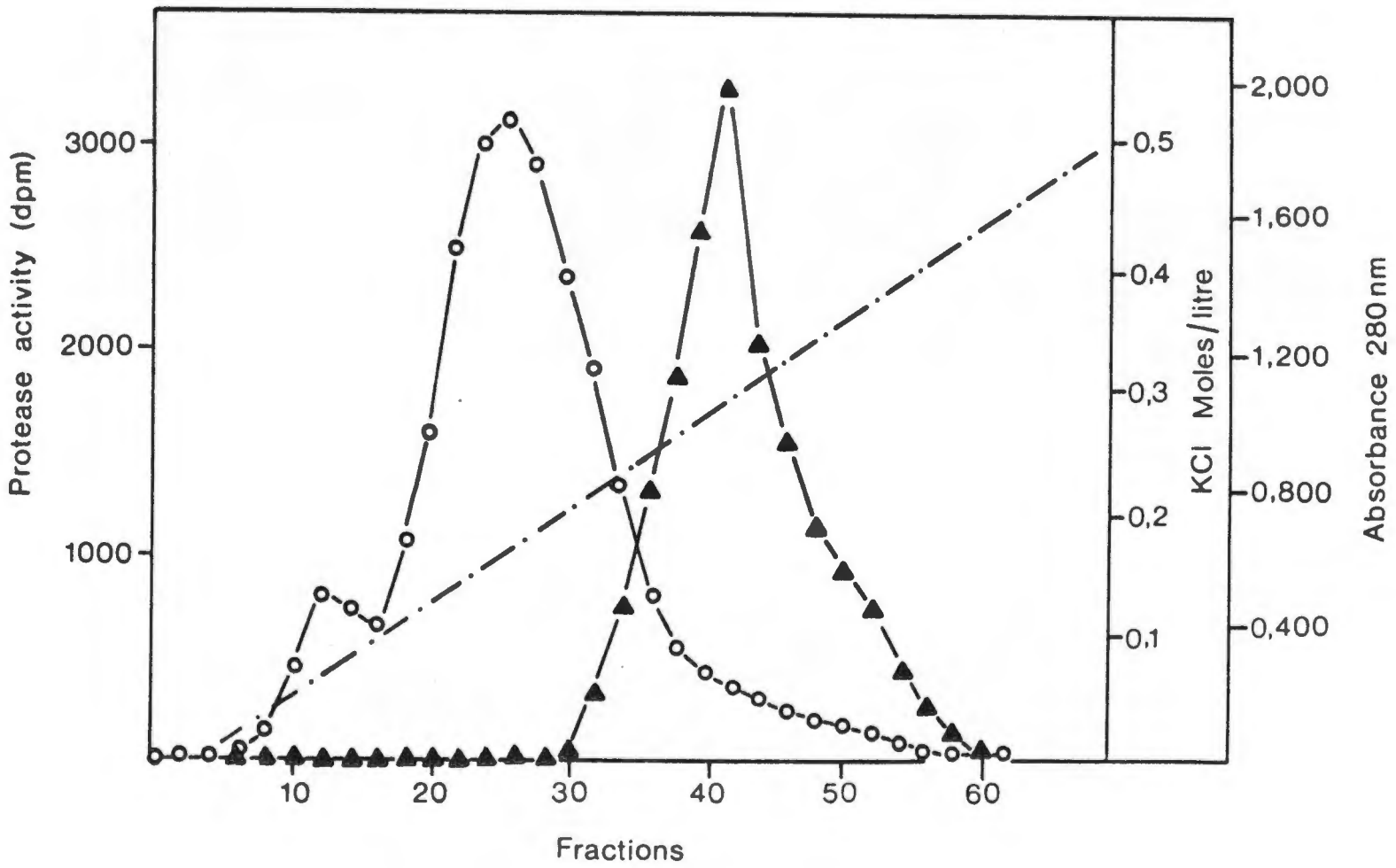
Agent		% Activation
$CaCl_2$	(1 mM)	0
DTT	(0,1 mM)	70
Cysteine	(1 mM)	40
ATP	(0,1 mM-1 mM)	5-25
Triton X100	1%	0
Triton X100 + ATP	(1 mM)	25
Imidazole	(25 mM)	25

salt gradient and all the protease activity appeared between KCl concentrations of 0.20 and 0.30 M (Fig.3.1). This activity peak (representing about 2% of the total protein applied to the column) was pooled and the protein precipitated overnight at 90% saturation with ammonium sulphate. The resulting pellet was taken up in 2 ml 50 mM Tris-HCl containing 0.5 mM DTT pH 7.5, and applied to a Sepharose 6B (or 4B) column (Fig. 3.2). The activity peak eluted with a K_{av} indicative of an aproximate molecular weight of 500 000. It should be noted that the ammonium sulphate step caused an appreciable loss of apparent enzyme activity which could not be recovered subsequently. Other methods of protein concentration (not described) caused a similar loss. The final apparent purification was 1000-fold over the dialysed HSSN; the yield through the two column steps was 95% (Table 3.1). (The question of the quantitativeness of the assay procedure is addressed below). The same K_{av} was obtained when the Sepharose 6B column was run at high ionic strength (0.6 M KCl) or in the presence of 0.5% Triton X-100, and the elution behaviour was also independent of the presence or absence of DTT. These findings suggest that the enzyme is genuinely of high molecular weight.

When the heat-step was omitted from the purification scheme, the Sepharose 6B (or 4B) protease activity profile revealed two peaks; one in the 500 000 molecular weight range (which was also routinely observed when the heat-step was included) and a second peak of activity in the excluded volume (V_o).

Fig. 3.1

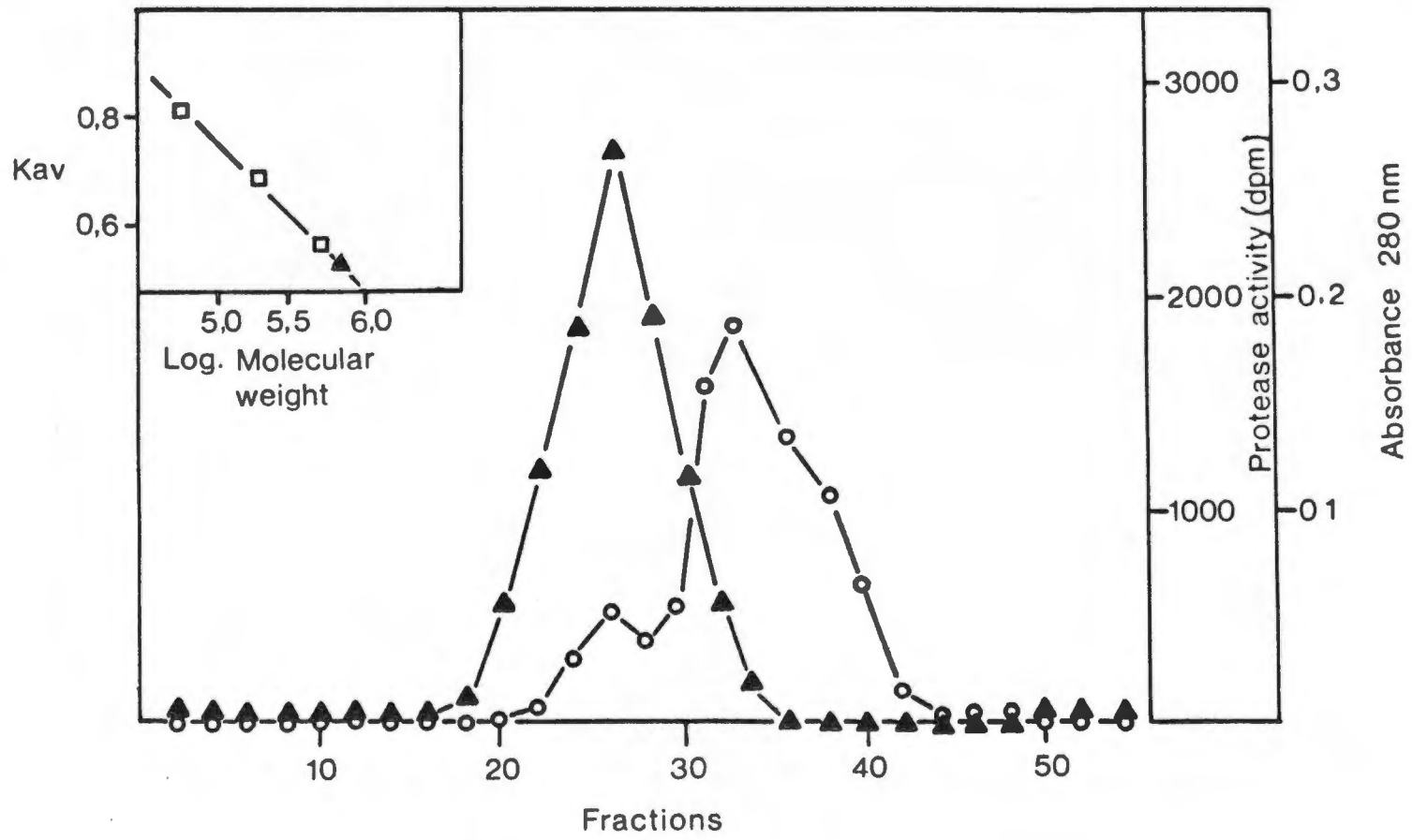
DEAE-CELLULOSE CHROMATOGRAPHY OF A SOLUBLE MUSCLE FRACTION



A high-speed supernatant obtained from 8 g of homogenized muscle was dialysed and subjected to heat-treatment (see Methods). It was then chromatographed on a DEAE-cellulose column as described in Methods, with a linear KCl gradient (·——·). Fraction volumes were 2 ml and the assays of protease activity (▲—▲) and absorbance measurements (o—o) were conducted as also described in Methods.

Fig. 3.2

SEPHAROSE 6B CHROMATOGRAPHY



A pooled and concentrated fraction containing all the protease activity from the DEAE-cellulose column was chromatographed on a Sepharose 6B column as described in Methods. Protease assays (▲——▲) and absorbance measurements (o——o) were conducted as described in Methods. Inset: Calibration of the column with the marker proteins ferritin, catalase and haemoglobin, in order of decreasing size.

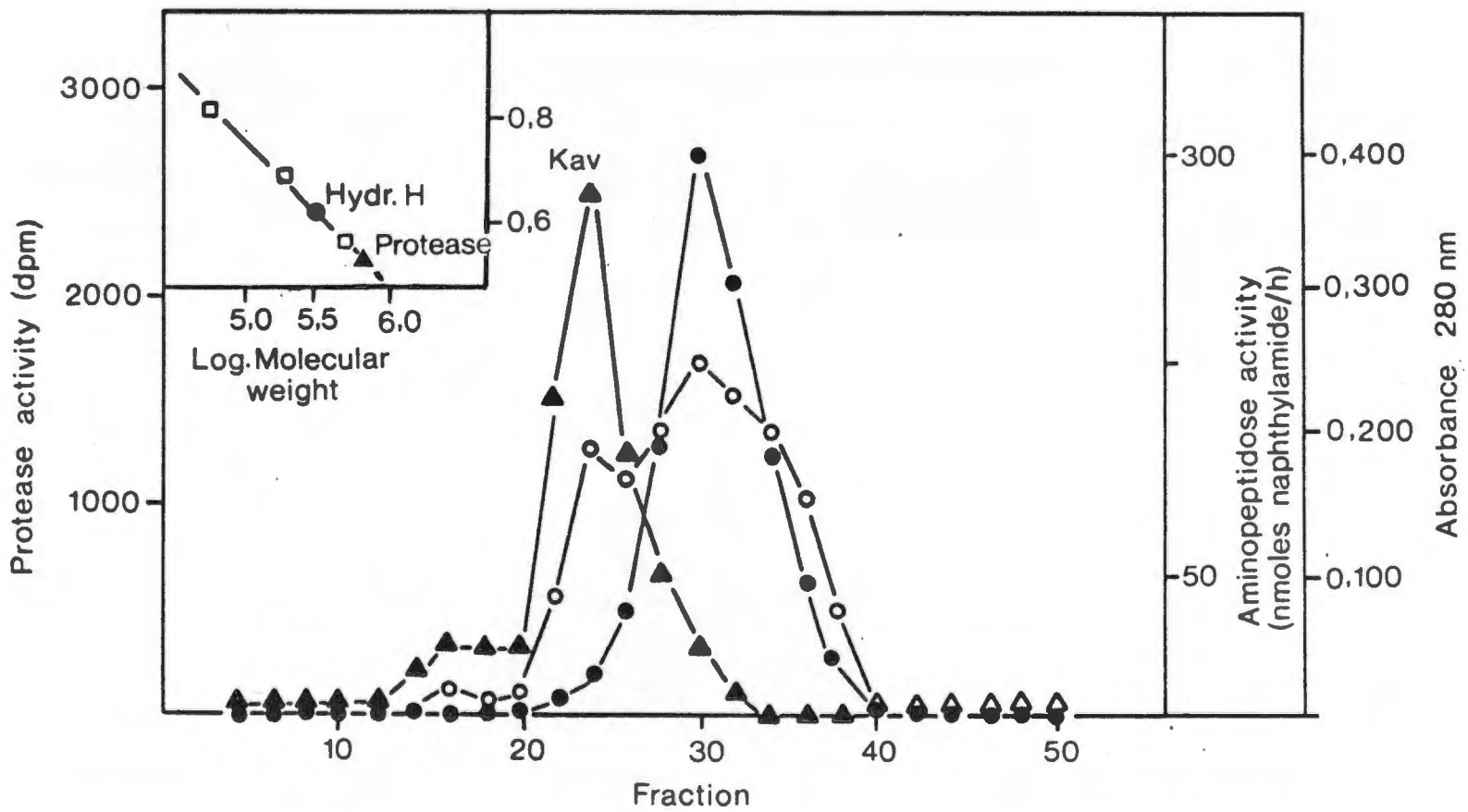
The latter activity was pepstatin-inhibitable, and had a pH optimum around 5.0; it presumably represented a protease (s) of lysosomal origin, probably cathepsin D. Treatment of this peak with Triton-X100 failed to produce a shift in its position of elution, indicating that this was not a membrane-associated protease activity.

3.3.2. Distinction of protease from aminoendopeptidase

The undialysed HSSN fractio catalysed significant hydrolysis of L Leu-N-Nap at pH 7.5. This aminopeptidase activity was also adsorbed to the DEAE cellulose column and eluted at 0.20 M KCl in a peak which overlapped extensively with the [^{14}C-methyl]-globinase activity. Measurement of enzyme activity in each of the fractions from the Sepharose 6B column against [^{14}C-methyl]-globin and the aminopeptide substrate gave two well-defined but separate peaks of activity - a 500 000 molecular weight peak of protease activity and a second enzyme, of approximate molecular weight 350 000, which degraded Leu-N-Nap (Fig. 3.3). The latter enzyme was partially inhibited by iodoacetate (10 mM) and by leupeptin (0.5 mM) and markedly inhibited by Zinc ions (Table 3.3). The optimal pH for this activity was 7.5 and the enzyme hydrolysed Bz-Arg-N-Nap less well than Leu-N-Nap. This entity was thus almost certainly the aminoendopeptidase, hydrolase H recently described in rabbit muscle extracts by Okitani *et al* (1981).

Fig. 3.3

SEPARATION OF AMINOPEPTIDASE FROM ENDOPROTEASE ON SEPHAROSE 6B



A pooled and concentrated fraction prepared by DEAE-cellulose chromatography was applied to a Sepharose 6B column as described in Methods. Fractions of 1 ml were collected and aliquots (100 µl) were assayed separately in each case with [^{14}C methyl]-globin and Leu-N-Nap as substrates, both as described in Methods. [^{14}C methyl]-globinase activity (▲——▲); Leu-naphthylaminase activity (●——●); absorbance at 280 nm (o——o).

TABLE 3.3

HYDROLASE H: EFFECTS OF INHIBITORS ON ACTIVITY OF HYDROLASE H

Aliquots of the pooled fractions from the Sepharose 6B column showing activity towards Leu-N-Nap were assayed in the absence and presence of protease inhibitors; activity towards Leu-N-Nap was determined as described in Methods.

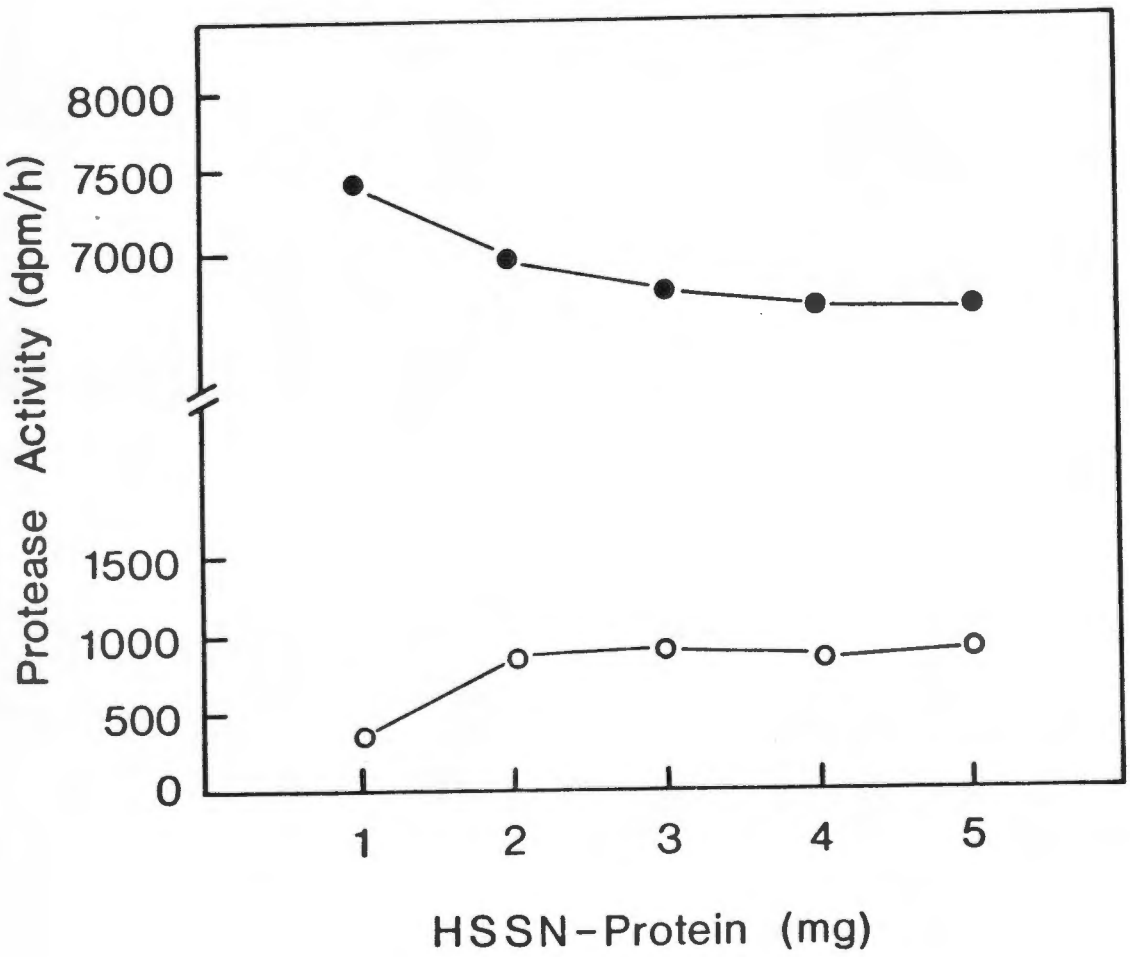
Inhibitor		Relative activity towards Leu-N-Nap
None		100%
Iodoacetate	(10 mM)	5%
$ZnSO_4$	(1 mM)	5%
Leupeptin	(20 µg/ml)	30%
	(40 µg/ml)	20%
Chymostatin	(20 µg/ml)	100%
	(40 µg/ml)	100%
Pepstatin	(20 µg/ml)	100%
	(40 µg/ml)	100%

3.3.3. Validity of the radiometric assay

It was noted that the protease activities of impure enzyme preparations were not linear with protein concentration, while the column purified material showed a linear relationship with increasing enzyme concentrations (Figs. 3.4, 3.5, and 3.6). A time course study over two hours revealed that the routine incubation period for the two assays was well within the period of constant rate of activity of the protease (Fig. 3.7). In addition, there was a large increase in activity caused by dialysis of HSSN preparations (see above). These phenomena could have been caused by endogenous inhibitors or by the presence of contaminating proteins 'competing' with the $|^{14}C|$-substrate. Accordingly, an undialysed HSSn fraction (2 ml volume) was directly applied to a Sepharose 6B column after which the peak with high protease activity was pooled. This enzyme was then assayed in the presence and absence of 100 µl aliquots of all the other inactive fractions to assess whether specific endogenous inhibitors were present, or whether native proteins competed randomly with the radioactive substrate in the routine assays. Decreases in protease activity correlated generally with the total amounts of protein added in other fractions, and there was no significant indication of a well-defined peak of inhibition (Fig. 3.8). (Precise superimposition of the protein and 'inhibition' curves was not possible in areas of activity overlap due to the protease peak). The results were thus in keeping with the idea that native proteins present in the extracts were all more or less effective substrates for the protease and

Fig. 3.4

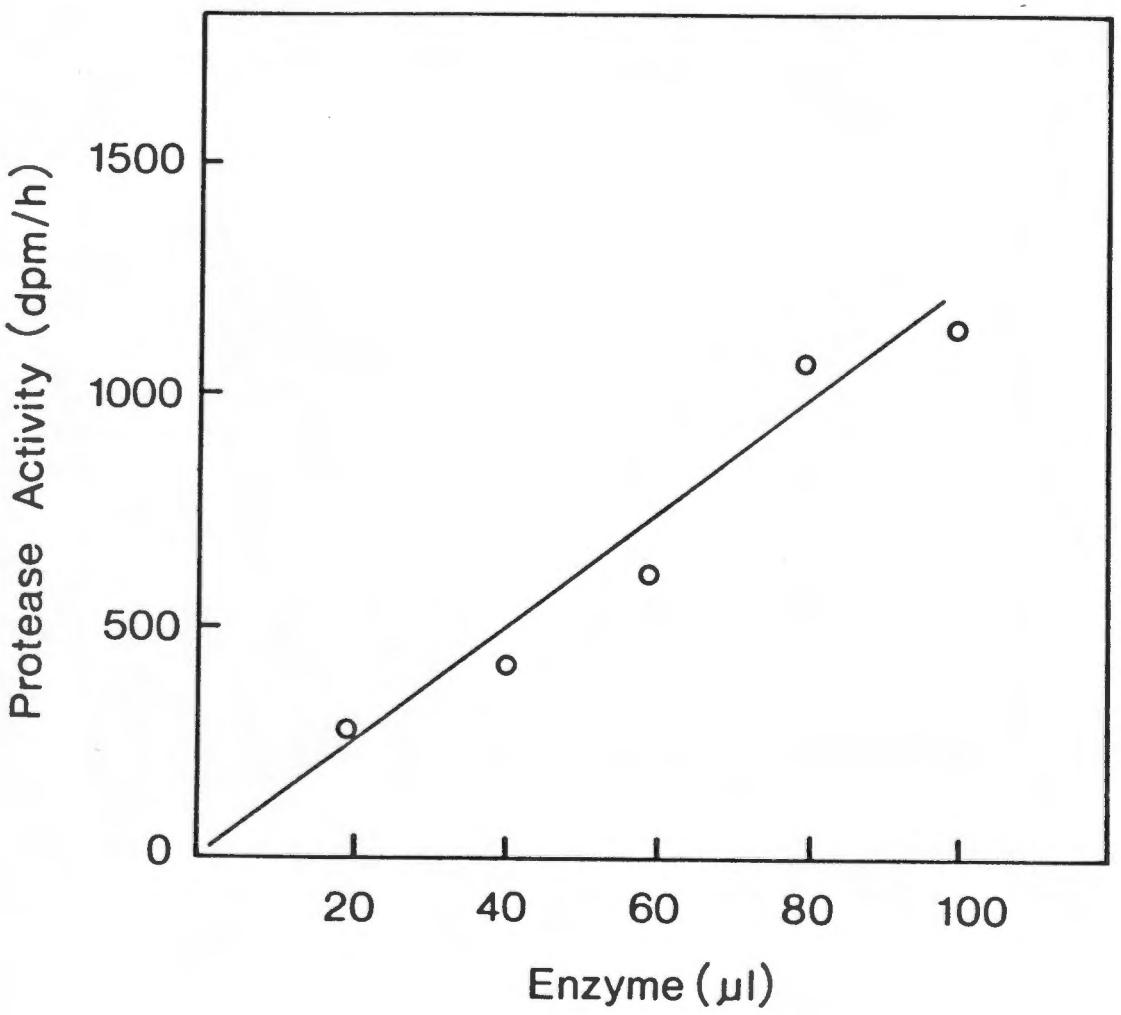
EFFECT OF INCREASING AMOUNTS OF HIGH-SPEED SUPERNATANT ON ACTIVITY OF PROTEASE(S)



Increasing amounts (protein) of dialysed (●—●) and undialysed (o—o) HSSN fractions were assayed with [^{14}C methyl]-globin; protease activity, determined as described in Methods.

Fig. 3.5

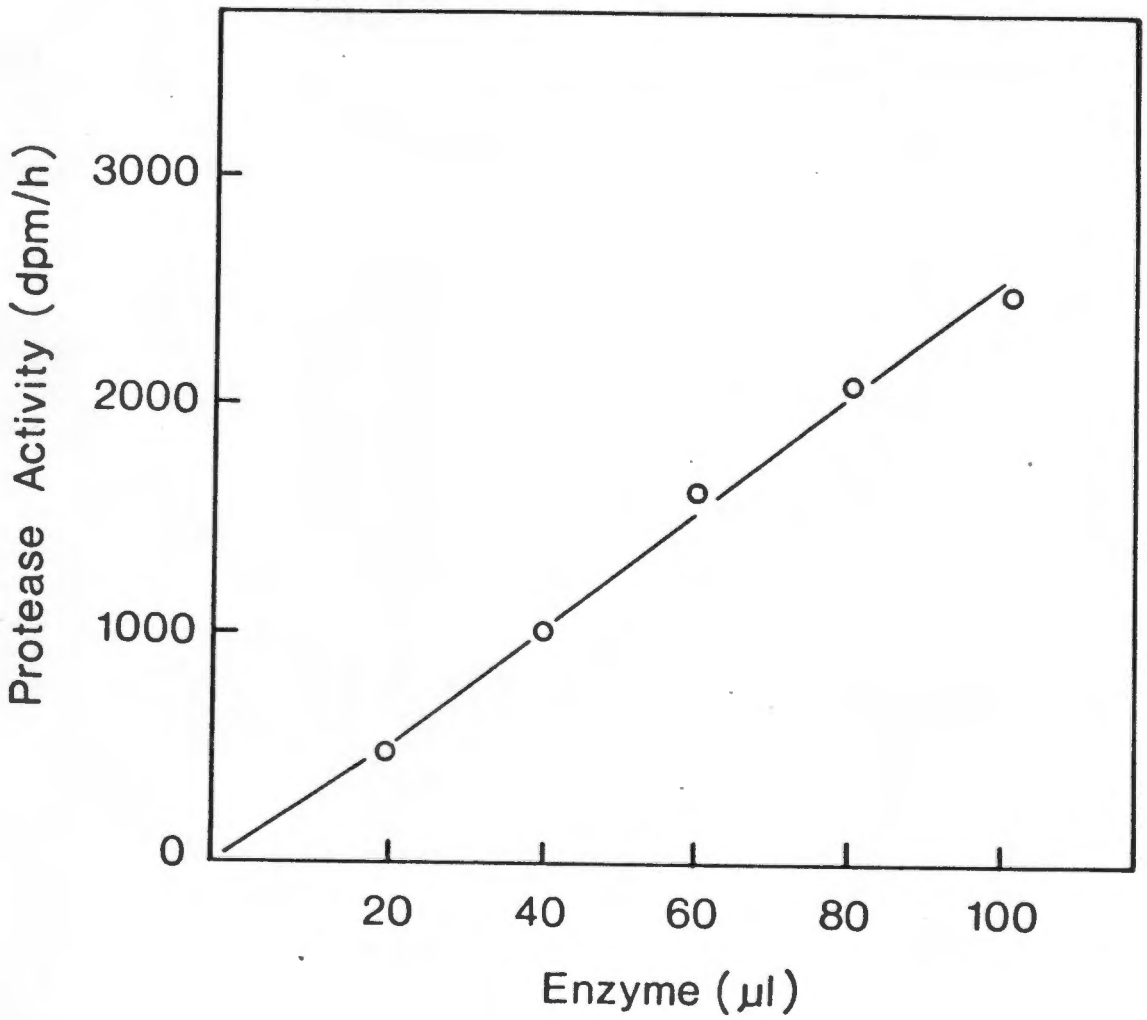
EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF DEAE CELLULOSE-PURIFIED PROTEASE



Increasing amounts (µl) of the DEAE cellulose-purified protease were assayed with [^{14}C methyl]-globin, as described in Methods.

Fig. 3.6

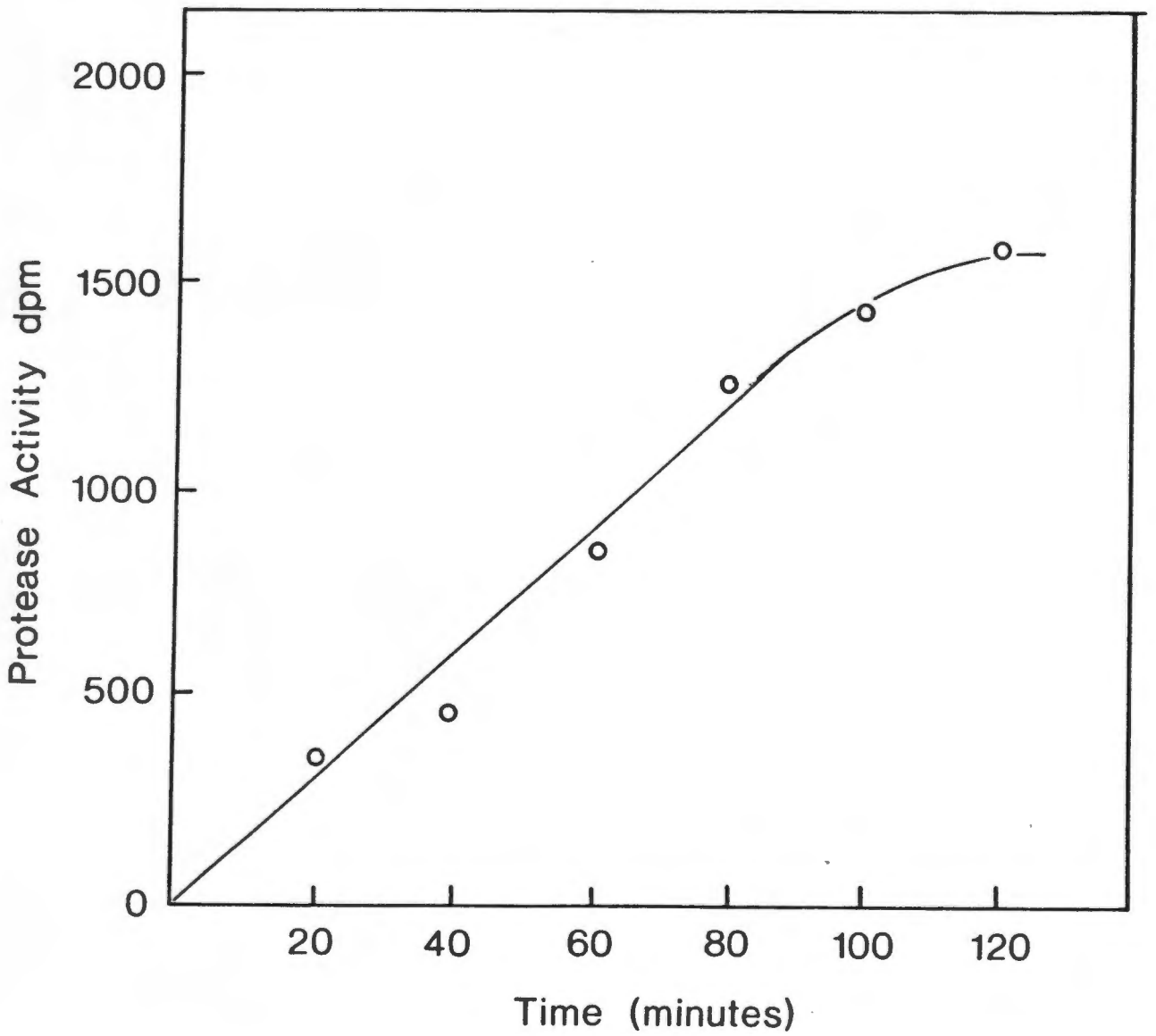
EFFECT OF ENZYME CONCENTRATION ON ACTIVITY OF SEPHAROSE 6B-PURIFIED PROTEASE



Increasing amounts (µl) of the Sepharose 6B-purified enzyme were assayed with [^{14}C methyl]-globin, as described in Methods.

Fig. 3.7

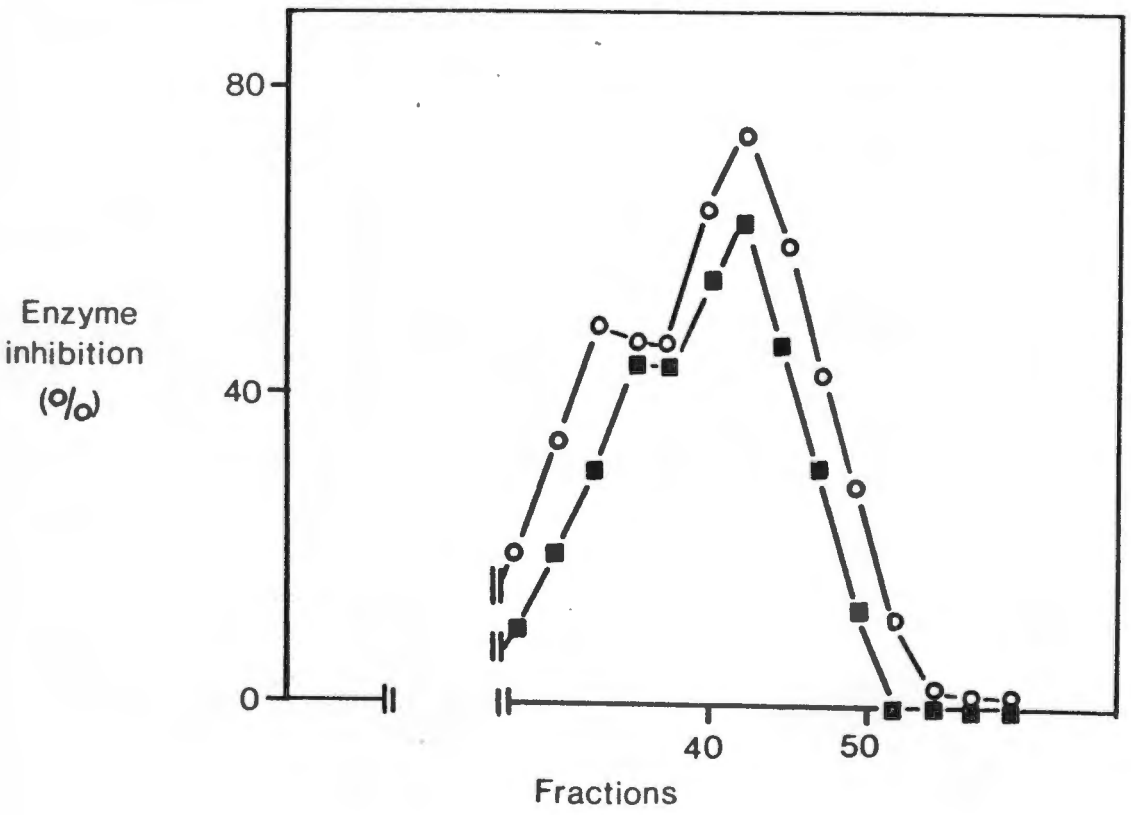
TIME COURSE OF PROTEASE ACTIVITY



The purified enzyme was assayed at 37°C and the TCA-soluble ^{14}C-fragments released from [^{14}C methyl]-globin at various time intervals measured as described in Methods.

Fig. 3.8

APPARENT ABSENCE OF A SPECIFIC INHIBITOR OF THE PROTEASE IN MUSCLE EXTRACTS



An undialysed HSSN fraction from 1 g of homogenized leg muscle was chromatographed on Sepharose 6B (0.9 x 60 cm) previously equilibrated with 50 mM tris-HCl, 0.5 mM DDT (pH 7.5), as described in Fig. 3.2. The protease peak was identified and pooled. Samples of this enzyme were assayed in the presence and absence of 100 µl aliquots of the whole range of other column fractions, and the percentage inhibition (if any) (■—■) was calculated in each case. Absorbance at 280 nm (o—o).

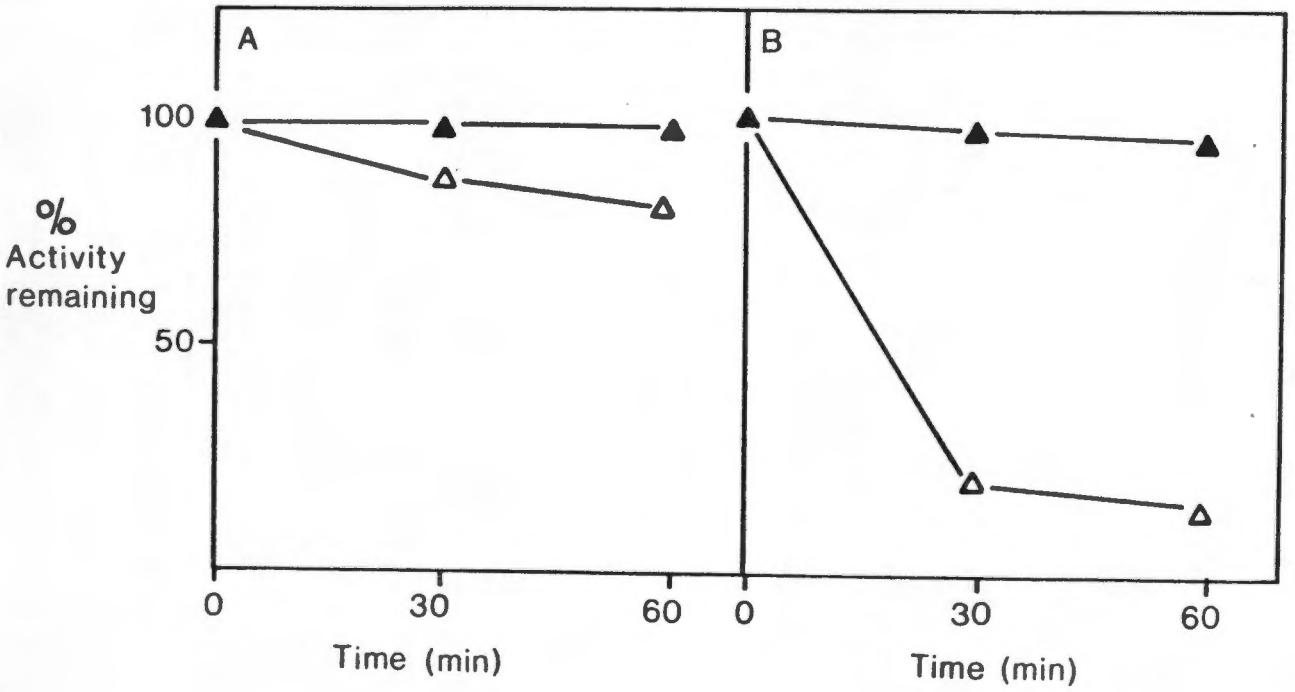
that no particularly effective inhibitor was present. It is noteworthy that no low molecular weight peak of inhibition was noted (V_t region of the column eluates). The activation caused by dialysis of the HSSN fractions (see above) cannot therefore be explained by ridding the fraction of low molecular weight protease inhibitor(s).

3.3.4. Effects of ATP

Enzyme activities throughout the purification procedure were measured in the presence or absence of 2 mM ATP and an ATP-regenerating system consisting of creatine phosphate and creatine kinase. Both the DEAE activity peak and the Sepharose activity peak showed a variable 'stimulation' in the presence of the ATP system. This inconsistent effect was further examined by pre-incubating the purified enzyme with and without the ATP system for 60 minutes at 37°C and 42°C, in the absence of exogenous substrate. Thereafter [^{14}C-methyl]-globin was added as well as ATP to those tubes lacking it, and the remaining activity was then assayed at 37°C for 60 minutes. The loss of activity was 20% when the pre-incubation was conducted at 37°C, and 90% at 42°C, in the absence of ATP. Pre-incubation in the presence of ATP markedly protected the activity (Fig. 3.9). ATP concentrations as low as 0.1 mM stabilized the protease to roughly the same extent. Citrate at 50 mM, creatine phosphate 20 mM, EDTA 1 mM and the non-hydrolysable ATP analogue, βγ-methylene ATP failed to stabilize the enzyme.

Fig. 3.9

EFFECT OF ATP ON THERMAL STABILITY OF THE PROTEASE



Samples of enzymes purified by column chromatography (Fig. 3.2) were pre-incubated at 37°C (A) and 42°C (B) for 30 and 60 min, in the presence (▲) and absence (△) of ATP. After this, the activity of the remaining enzyme was assayed at 37°C as described in Methods.

3.3.5. Other properties

The pH range of the protease with radioactive globin extended from 6.5 to 9.0 but the optimum was 7.5 (Fig. 3.10).

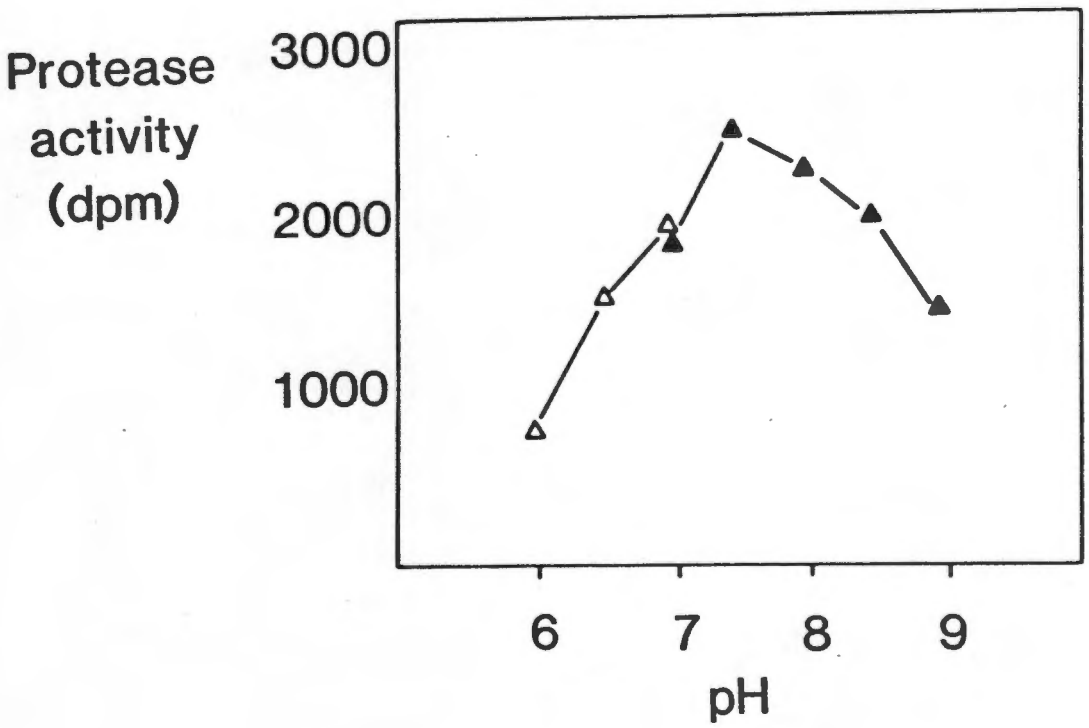
The purified enzyme was unstable in the frozen state, losing 80% of its activity within 4 days of freezing. However, when the enzyme was kept at $4^{o}C$, no loss of activity was observed over the first seven days, following which a gradual diminution in activity was noted, reaching 60% of the initial activity at 14 days.

The protease activity eluted ahead of ferritin from Sepharose 4B or 6B columns (calibrated with marker proteins at high molecular weight), at a position consistent with a molecular weight of 500 000 for the native complex (Fig. 3.2).

The protease appeared to contain an essential thiol group, since dithiothreritol and cysteine addition enhanced its activity, while N-ethylmaleimide (NEM) and p-chloromercuriphenylsulphonic acid (PCMPS) were inhibitory (Table 3.2). Chymostatin, leupeptin and pepstatin had no effect at concentrations known to inhibit the activities of entities such as the mast cell serine protease (Libby and Goldberg, 1980), Ca^{++}-activated protease (Reville *et al*, 1976) and the lysosomal acid hydrolases cathepsins B, H, L and D (Barrett, 1977). $ZnCl_2$ (1 mM) markedly inhibited the enzyme and imidazole (25 mM), a zinc chelating agent, caused a moderate increase in activity (Table 3.2).

Fig. 3.10

pH OPTIMUM OF PROTEASE



An enzyme preparation obtained as shown in Fig. 3.2 was incubated for 60 min at 37°C with [^{14}C methyl]-globin, in 25 mM imidazole-HCl buffer (pH 6-7) and tris-HCl buffer (pH 7-9); labelled TCA-soluble products were estimated as described in Methods.

The protease appeared to be an endopeptidase since analysis of the TCA-soluble products on Sephadex G25 revealed radioactive peptide ranging in molecular weight from 1500 to 15000 (Fig. 3.11).

3.3.6. Substrate selectivity

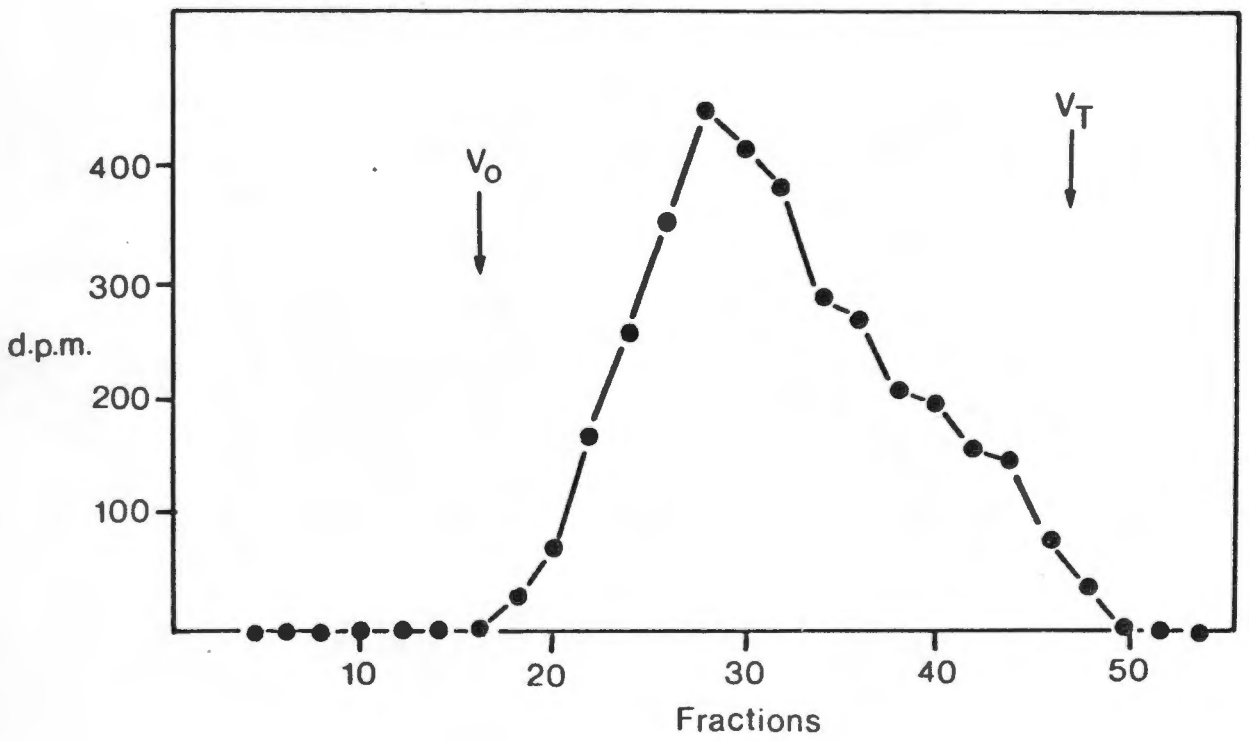
In addition to [^{14}C methyl]-globin, which may be considered to be a denatured but soluble protein, the enzyme preparation attacked denatured casein and azocasein, because addition of these proteins to the assay markedly diminished the production of radioactive products from the globin substrate (Fig. 3.12). Addition of bovine serum albumin did not "dilute" the product radioactivity in standard assays and this protein may thus not be attacked by the endoprotease.

In addition, the activity of a sample of hexokinase assayed by the method of Crabtree and Newsholme (1972) was significantly inactivated (>50%) when it was pre-incubated for 60 min at 37^{o}C with 10 μg of the high molecular weight protease (Fig. 3.13).

[^{14}C NEM]-labelled myofibrillar proteins were also attacked by the protease, but this substrate was not routinely used because the amounts of TCA-soluble fragments formed were small. [^{125}I]-labelled myofibrillar proteins were more susceptible to attack by the protease, but the background activity in this case was higher and the half-life of the label was shorter. For these reasons [^{14}C methyl]globin was used for routine assays.

Fig. 3.11

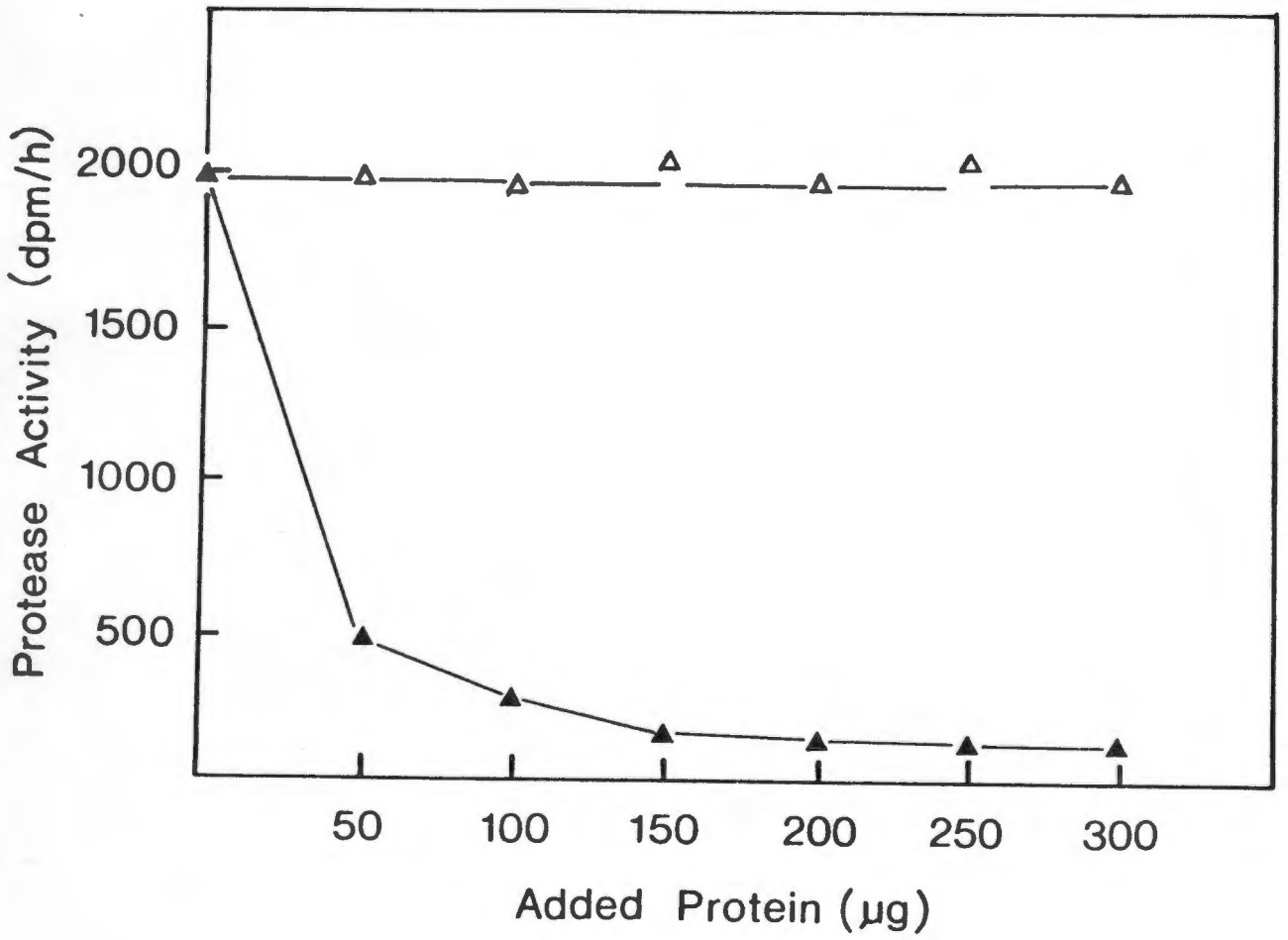
SIZE ANALYSIS OF PROTEASE DIGESTION PRODUCTS



A standard assay mixture constituted as described in Methods and containing enzyme purified as in Fig.3.2, was incubated and stopped by addition of TCA. After centrifugation, the supernatant was chromatographed on Sephadex G25, and radioactivity in the fractions (0,4 ml each) was measured, all as described in Methods.

Fig. 3.12

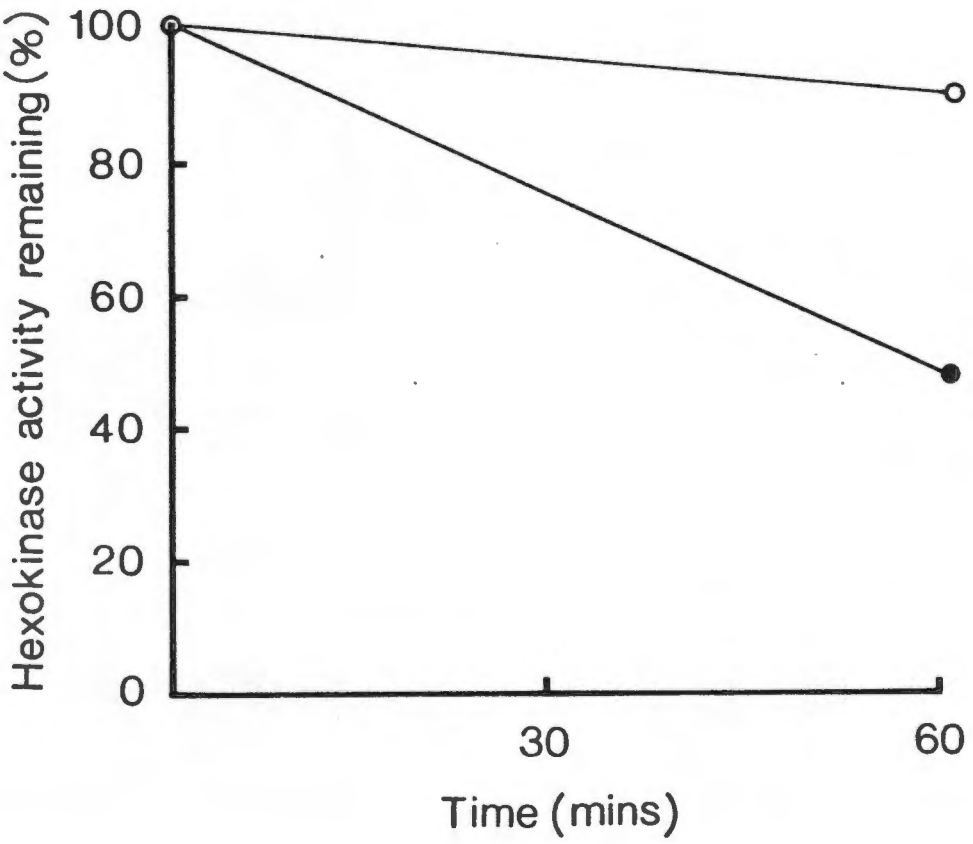
EFFECTS OF ADDITION OF CASEIN OR BOVINE SERUM ALBUMIN IN THE ACTIVITY OF THE PURIFIED ENZYME



The column-purified protease was assayed in the absence and presence of varying amounts of bovine serum albumin (Δ——Δ) or casein (▲——▲); protease activities were measured as described in Methods.

Fig. 3.13

HEXOKINASE INACTIVATION



Fresh rat skeletal muscle homogenate containing a known amount of hexokinase activity was incubated in the presence of 10 µg bovine serum albumin (o) or in the presence of 10 µg purified high molecular weight protease (●) at 37°C for 60 minutes, after which the remaining hexokinase activity was determined as outlined in Methods.

3.3.7. Proteolytic system in chicken reticulocytes

The reticulocyte lysate contained significant ^{14}C-methyl globinase activity at neutral pH, but this was not enhanced by ATP (Table 3.4). These results were unlike those of Ciechanover *et al* (1978), where the protease activity in the rabbit reticulocyte lysate was markedly enhanced in the presence of ATP.

Fraction I contained no detectable protease activity, while fractionation of the DE-adsorbed material (Fraction II) on Sepharose 4B revealed an activity peak in the 500 000 mol. wt. range and a smaller peak in the excluded volume (V_o) (Table 3.4). The activity of fraction II was not enhanced by ATP and addition of fraction I to fraction II did not result in ATP-dependent cooperativity (Table 3.4).

3.3.8. Absence of ubiquitin effect

Crude preparations of fraction I (ubiquitin) from rat skeletal muscle, rat erythrocytes and human erythrocytes (obtained by the method of Ciechanover *et al*, 1980[a]) did not show ATP-dependent complementation when assayed with reticulocyte fraction II. Similar attempts at complementation between ubiquitin and the high molecular weight rat muscle protease at various levels of purification, in the presence and absence of ATP, did not show any of the features of a multi-component, ATP-dependent system.

TABLE 3.4

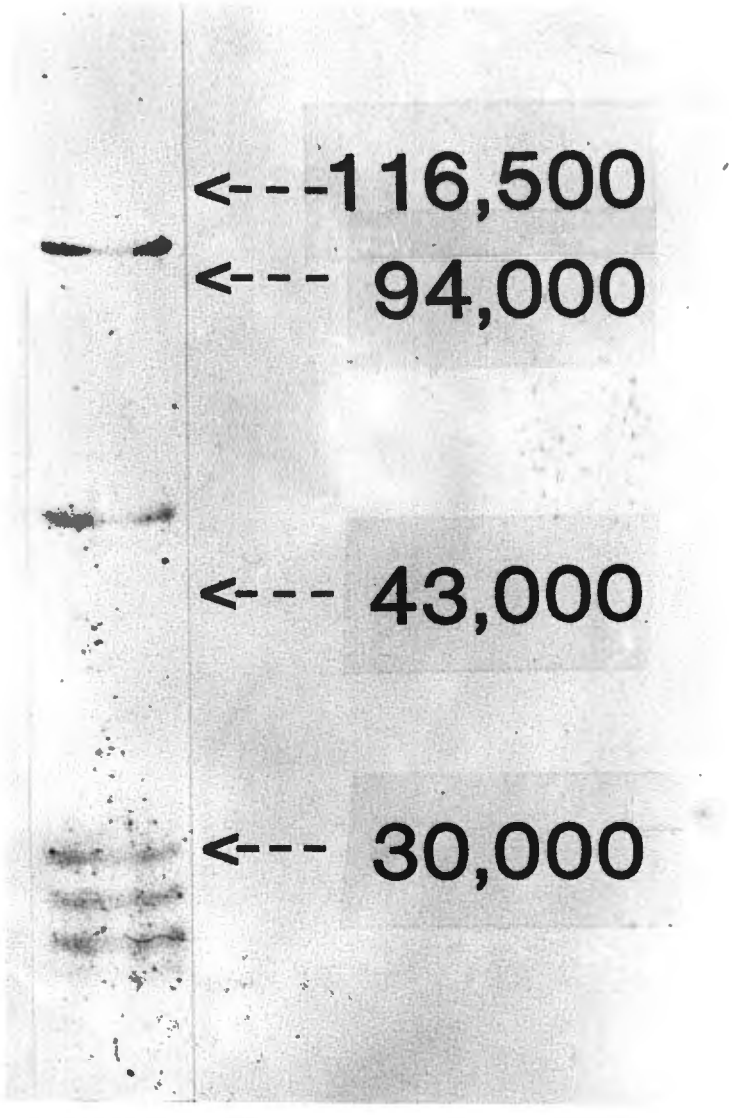
PROTEASE ACTIVITIES IN CHICKEN RETICULOCYTE EXTRACTS

Protease activities (determined in the presence and absence of ATP) were measured in reticulocyte extracts (at all the levels of purification), all as described in Methods. Reconstitution of the fractions was also studied to detect possible ATP-dependent complementation.

	Activity dpm/h	
	- ATP	+ ATP
Reticulocyte lysate	6000	5200
Fraction I	0	0
Fraction II	6500	5800
Fractions I + II	4500	4000
V_o Peak (Seph 4B)	1500	1000
$V_{500000\ m.wt.}$ Peak (Seph 4B)	5000	5500

Fig. 3.14

SDS-POLYACRYLAMIDE GEL ELECTROPHORETIC ANALYSIS OF THE ENDOPROTEASE



An aliquot of the purified endoprotease from the Sepharose 6B column was concentrated and analysed on a SDS-polyacrylamide slab gel (see Methods). The molecular weights of various protein standards are indicated in the figure.

Although no complementation was obtained in the case of rat muscle and chicken reticulocyte extracts, a high molecular weight protease (∿500 000 daltons) was isolated from each of these extracts. These proteases all had similar molecular masses, and also shared other properties (e.g. thermal stabi lization by ATP).

3.3.9. Subunit structure of endoprotease preparation

Analysis of the purified enzyme by electrophoresis on a SDS-polyacrylamide slab gel revealed two subunits with approximate molecular weights of 100 000 and 52 000 representing most of the protein (Fig. 3.14). A number of bands in the molecular weight region between 24 - 30 000 were also present.

3.4. DISCUSSION

The most active protease activity 'detectable' at neutral or mildly alkaline pH in rat skeletal muscle extracts is a chymotrypsin-like serine protease (chymase) (Mayer et al, 1974). This protease, which appears to co-precipitate with myofibrillar proteins at low salt concentrations, is solubilized at high ionic strengths and in this state is optimally active against myofilaments as well as against exogenous substrates (e.g. casein). The protease is chymostatin-sensitive and its activity disappears from tissues in which the mast cells have been degranulated (McKee et al, 1979; Libby and Goldberg, 1980; Edmund and Pennington, 1981).

The soluble component of liver extracts, prepared in low ionic strength buffers, contains a high molecular weight alkaline protease (Rose et al, 1979; De Martino and Goldberg, 1979); more recently, a large enzyme called Hydrolase H has also been isolated from rabbit skeletal muscle by Okitani et al (1981). In addition, Hershko et al have identified a high molecular weight protease, in reticulocyte extracts, which forms part of a multi-component system for the degradation of denatured globin in an ATP-dependent manner (Hershko et al, 1979, 1980, 1981). All these activities are much less active than the chymase with respect to most protein substrates tested.

The data presented here concern the properties and purification of a 500 000 molecular weight protease in the membrane-free fraction of skeletal muscle homogenates. The enzyme is optimally active at a pH of 7.5 and is not inhibited by a range of Umezawa antibiotic inhibitors nor stimulated by Ca^{++}, even in the presence of EGTA. This latter finding eliminates the possibility that one of the Ca^{++}-requiring thiol protease is involved, and leupeptin inhibition shown by such enzymes was also absent (Dayton et al, 1981). Other enzymes which are excluded from consideration are chymase (chymostatin-inhibited), the aminopeptidase Hydrolase H (Okitani et al, 1981) which is inhibited by leupeptin (and was separated from thehigh molecular weight protease) and the various cathepsins B, D, L and H (Barrett, 1977). Stimulation by thiol agents and inhibition by PCMPS and NEM point to an active thiol group.

ATP stimulation of protease activity in enzyme fractions at various levels of purification was variable and slight. However, the heat stability of the enzyme at 42°C was very significantly enhanced by the nucleotide even at concentrations as low as 0.1 mM, indicating that the enzyme had some kind of binding site for ATP. The ATP-stabilized liver protease studied by Rose et al,(1979) was also stabilized by citrate; this polyanion was ineffective in preventing the thermal denaturation of the enzyme here described.

Nevertheless, the muscle-derived enzyme had several other properties in common with the liver enzymes described by De Martino and Goldberg (1979) and by Rose et al (1979). Thus its pH optimum was also between 7 and 8, and the activation by thiol compounds, inhibition by NEM and PCMPS, heat stabilization by ATP and endoprotease character were all similar. This enzyme differed from that of De Martino and Goldberg (1979), however, in that their enzyme showed a more marked inhibition caused by iodoacetate (mild or absent in the enzyme here reported), a marked stimulation (almost 100%) caused by ATP (slight and variable in this case) and enhancement of ATP dependence by addition of the non-ionic detergent, Triton X-100 (absent in this case). Again the high molecular weight (450 000) protease characterized in reticulocyte extracts requires ATP for thermal stabilization and participates together with ubiquitin in a multi-component, ATP-dependent system, that catalyses the selective degradation of denatured globin to the level of amino acids. It is noteworthy that

albumin is attacked by the reticulocyte system while the muscle enzyme did not apparently act on this protein. Since βγ-methylene ATP failed to stabilize the muscle protein, a possible role for ATP hydrolysis may eventually be found for a system of which this high molecular weight protease may be a part.

There appear to be important differences between the high molecular weight proteinase here described and that isolated by Hardy et al (1981) from human skeletal muscle. Examples are the rapid action of the former on casein and globin, the absence of aminopeptidase activity, lack of Ca^{2+} stimulation, marked DTT stimulation, and absence of inhibition by ATP. No data are available in respect of the powerful Zn^{2+} inhibition and the heat-stabilization afforded by ATP, which are features of the enzyme outlined in this report.

The crude muscle enzyme briefly described by Etlinger and co-workers (1981) displays marked activation by ATP which is diminished by haemin addition, and is apparently dependent on a ubiquitin-like fraction also obtained from muscle; no other details are available. Another cysteine proteinase isolated from smooth muscle has been found to have a very large size (1300 000) and a mildly alkaline pH optimum (Roth et al, 1981). Both casein and albumin are attacked and Zn^{2+} inhibits weakly (30% at 1 mM); no effects of ATP were reported, either in terms of activation or stabilization of the very heat-labile enzyme.

No specific inhibitor was detected in the high-speed supernatant fraction, but endogenous native proteins competed with the denatured radiolabelled substrate for degradation. The role of the enzyme is thus not limited to the degradation of "abnormal" or partially denatured proteins, as appears to be the case in the reticulocyte system (Hershko *et al*, 1980).

A high molecular weight, cysteine endopeptidase from rat skeletal muscle present in the particle-free fraction of muscle extracts and optimally active at pH 7.5 has thus been characterized. Because of the significant thermal stabilization of the enzyme caused by ATP, this protease may form part of a multi-component ATP-dependent proteolytic system similar to that reported in reticulocytes (Hershko *et al*, 1979, 1980, 1981; Ciechanover *et al*, 1980, 1981).

CHAPTER 4

THE RELATION BETWEEN *IN SITU* PROTEOLYSIS IN SKELETAL MUSCLE AND THE *IN VITRO* ACTIVITY OF CERTAIN PROTEASES

4.1. INTRODUCTION

The attempts of biochemists to understand the basis of protein degradation have so far resulted in the isolation and characterization of several lysosomal and non-lysosomal proteases, in the case of many species of animals and a variety of muscle types. Data on the interaction of such entities with endogenous inhibitors have also permitted concepts of controlled proteolysis to develop. An extension of these approaches is the correlation of *in situ* rates of protein degradation with assayable activities of proteases in the case of physiological or pathological perturbations. Such experiments are based on the concept of a rate-limiting enzyme step for the overall degradative process in skeletal muscle. In essence, the most decisive advance in this field will be the identification of such a critical protease(s) and the determination of regulatory mechanisms which control its activity.

One therefore has set out to measure *in situ* proteolytic rates of isolated, intact soleus (red) and extensor digitorum longus (white) skeletal muscles derived from control, denervated, starved, diabetic, hyperthyroid and cortisol-treated animals, and also from rats in whom mast cell degranulation had been produced by treatment with compound 48/80. In each case a comparison was made between *in situ* proteolytic rates and the *in vitro* activities of chymase as well as the high molecular weight protease.

4.2. MATERIALS AND METHODS

4.2.1. Preparation of rats.

Long-Evans hooded rats (100 gm body mass) were injected intra-peritoneally with an anaesthetic dose of pentobarbitone sodium and sacrificed by decapitation. The rats were maintained, before experimentation, on standard laboratory chow and were injected intra-peritoneally with various agents (see below) to alter degradation rates.

4.2.2. Physiological manipulation to enhance protein degradation.

(a) Hyperthyroidism: Rats received catabolic doses of L-tri-iodothyronine (Carter et al, 1981) 25 µg/100 gm body wt., injected daily intraperitoneally over 14-18 days.
(b) Denervation: Unilateral sciatic nerve denervation was performed (Goldspink, 1976) with the contra-lateral hind-limb being the control. (c) Diabetes Mellitus: Diabetes was induced (Mayer et al, 1974) with alloxan (16 mg/100 gm) injected intra-peritoneally as a single dose and rats sacrificed on Day 4. (d) Starvation: This was carried out for a 48 h period. (e) Hypercortisolism: Hydrocortisone (5 mg/100 gm) was injected daily intra-peritoneally for 5 days prior to sacrifice (Goldberg, 1980).

4.2.3. Mast cell degranulation.

Degranulation of mast cells was induced according to the method of Pastan and Almqvist (1966) and Riley (1959) - see Chapter 2.

4.2.4. In vitro enzyme activities.

Hamstring muscles (0,5 gram) from the various rats were dissected out, freed of fat and connective tissue and homogenized in 5 volumes of 50 mM Tris-HCl, 0,5 mM DTT pH 7,5.

A high speed supernatant fraction (HSSN) and a myofibrillar, sedimentable fraction (MF) were prepared as described in Chapters 3 and 2, respectively.

The various HSSN fractions were applied to Sepharose 6B columns (60 x 0,9 cm), previously equilibrated with the homogenization buffer, the fractions containing the activity peak against [^{14}C]methylglobin (for details of assay conditions see Chapter 3) were pooled and the activity of this pooled peak determined and expressed as total and specific activity. Lowry proteins were measured with bovine serum albumin as standard (Lowry 1951). These activities represented the high molecular weight protease activity in these HSSN fractions.

Myofibril-associated protease ('Chymase') activities were determined at high ionic strength (for details see Chapter 2), and expressed as nmoles tyrosine/mg MF protein/3 h.

4.2.5. Tissue incubations.

Net rates of proteolysis in intact extensor digitorum longus (EDL) and soleus muscles were assessed generally according to the method prescribed for rat diaphragm by Fulks et al (1975). A preincubation for 30 minutes was

followed by incubation of the muscles for 2 h in Krebs-Ringer bicarbonate buffer gassed with 95% O_2/5% CO_2. Incubations were terminated with TCA (10% final concentration), the samples were centrifuged, and the tyrosine contents of the supernatants determined fluorimetrically (see above). Cycloheximide (0,5 mM) was added to some systems to test whether there was a need to block re-utilization of amino acids for protein synthesis in order to obtain accurate values for overall degradation rates.

4.2.6. Materials.

Dithiothreitol (DTT), EGTA, Compound 48/80 and L-triiodothyronine were obtained from Sigma Chemical Co. All other chemicals used were of Analytical Reagent grade.

4.3. RESULTS

4.3.1. In situ proteolysis

Rates of proteolysis in intact EDL (white fibre type) and soleus (red fibre type) muscles from fed normal rats were not significantly different in the presence or absence of cycloheximide.

Pretreatment for 5 days with daily injections of Compound 48/80, the mast cell degranulator, did not cause any alteration in the basal rates of *in situ* proteolysis (Table 4.1), and the inhibition of these rates caused by chymostatin and leupeptin was of the same order in muscles derived from rats

TABLE 4.1

IN SITU PROTEOLYSIS

Protein degradation rates in intact skeletal muscles from control, denervated, compound 48/80-treated and denervated (with or without 48/80-treatment) rats were determined as described in Methods. Duration of denervation was four days before sacrifice in normal fed rats, while in 48/80-treated rats, sciatic nerve denervation was carried out on the fourth day of treatment and this treatment was continued for a further four days before sacrifice. Chymostatin and Leupeptin were tested at concentrations of 20 µg/ml in each case-

	PROTEOLYTIC RATES (nmoles tyrosine/mg/2 h)			
	NORMAL	DENERVATED	48/80 TREATMENT	DENERVATED + 48/80 TREATMENT
Soleus	0,32	0,55	0,31	0,56
+ Chymostatin (% Inhibition)	0,22 (30%)	0,34 (30%)	0,20 (35%)	0,38 (32%)
+ Leupeptin (% Inhibition)	0,19 (40%)	0,30 (45%)	0,18 (42%)	0,29 (48%)
EDL	0,22	0,58	0,21	0,59
+ Chymostatin (% Inhibition)	0,14 (35%)	0,38 (35%)	0,15 (30%)	0,38 (36%)
+ Leupeptin (% Inhibition)	0,13 (40%)	0,31 (47%)	0,12 (45%)	0,33 (44%)

subjected to mast cell - degranulation, compared with matched controls.

Furthermore, denervation resulted in enhanced proteolytic rates of the same magnitude in the case of normal rats as was found in the case of rats after mast cell degranulation, suggesting that the enhanced rates of proteolysis caused by denervationwere not mediated by changes in chymase activity. With the exception of control, 48/80 treated and hydrocortisone-treated rats, all other groups (diabetes mellitus, hyperthyroidism, denervation, starvation) were associated with enhanced rates of proteolysis in the case of both white and red muscle fibre types studied (Tables 4.2 and 4.3).

It was noted that four days' denervation resulted in greater increase in proteolytic rates in EDL compared with soleus muscles (Table 4.1), while the soleus lost more weight (Table 4.4) - indicating that the greater loss in mass undergone by red muscle may have been due to an associated reduction in protein synthesis. By concomitant measurement of protein synthesis and degradation in such intact isolated muscles, Goldspink (1976) has shown that while the EDL is accompanied by greater degradation following denervation, the soleus undergoes less protein synthesis (at 4 days' denervation), accounting for these mass changes.

The ratio of heart weight/body weight progressively decreased with increasing body weight in all groups studied, with the exception of the hyperthyroid animals, where this

TABLE 4.2

PROTEIN DEGRADATION IN INTACT MUSCLES AND "MYOFIBRILLAR PROTEASE" ACTIVITY

Protein degradation rates in intact soleus and EDL muscles from control and treated rats were determined as described in Methods. MF fractions from hindlimb muscles of the same rats were prepared and the MF protease activities determined as described in Methods.

GROUP	PROTEIN DEGRADATION (nmoles tyrosine/mg/2 h) Expt. No. = 4 (± S.D.)		MF-PROTEASE ACTIVITY (nmoles tyrosine/mg/3 h)
	Soleus	EDL	
Control	0,31 (±0,06)	0,23 (±0,02)	56 (±3)
Compound 48/80-treated	0,30 (±0,02)	0,21 (±0,02)	3 (±0,8)
Hypercortisolism	0,32 (±0,04)	0,23 (±0,03)	53 (±3,6)
Denervation	0,55 (±0,08)	0,56 (±0,11)	56 (±5)
Starvation	0,87 (±0,08)	0,69 (±0,19)	55 (±4)
Hyperthyroidism	0,49 (±0,02)	0,37 (±0,02)	56 (±3,4)
Diabetes Mellitus	0,47 (±0,10)	0,30 (±0,06)	55 (±4,1)

TABLE 4.3

PHYSIOLOGICAL MODULATION OF PROTEOLYTIC RATES IN SKELETAL MUSCLES: HIGH MOLECULAR WEIGHT PROTEASE ACTIVITIES

Proteolytic rates were measured in intact soleus and EDL muscles, as described in Methods. The high molecular weight protease activities in hindleg skeletal muscles dissected from control, compound 48/80-treated, starved, hyperthyroid, diabetic and denervated rats, were determined in (unheated) high-speed supernatant preparations subjected to Sepharose 6B chromatography as also described in Methods.

		PROTEOLYTIC RATES IN INTACT TISSUE (nmoles tyrosine/mg/2h)		PROTEASE ACTIVITIES	
		Soleus	EDL	Total (dpm/min)	Specific (dpm/mg/min)
Control	Expt. No. 1	0,31	0,23	1380	27000
	2	0,30	0,21	1200	26000
	3	0,32	0,24	1250	25000
48/80 Treated	Expt. No. 1	0,29	0,22	1100	27000
	2	0,28	0,21	1400	27500
	3	0,30	0,20	1300	25000
Starvation	Expt. No. 1	0,87	0,64	1000	22000
	2	0,92	0,61	1300	29000
	3	0,85	0,65	1350	27500
Hyperthyroidism	Expt. No. 1	0,49	0,37	1300	28000
	2	0,47	0,39	1500	25000
	3	0,48	0,35	1700	30000
Denervation	Expt. No. 1	0,55	0,56	1200	27000
	2	0,59	0,60	1350	26500
	3	0,52	0,55	1600	29500
Diabetes Mellitus	Expt. No. 1	0,47	0,33	1250	26000
	2	0,44	0,35	1400	28500
	3	0,42	0,32	1100	25000
Hypercortisolism	Expt. No. 1	0,30	0,22	1300	23000
	2	0,35	0,25	1150	27000
	3	0,32	0,21	1450	24500

TABLE 4.4

% MASS CHANGE IN SOLEUS AND EDL MUSCLES IN THE TREATED ANIMAL GROUPS COMPARED WITH MATCHED CONTROLS

The masses of intact soleus and EDL muscles in the animal groups studied were compared after complete dissection with those of matched controls.

	SOLEUS	EDL
Starvation	- 40%	- 37%
Denervation	- 21%	- 12%
Diabetes Mellitus	- 14%	- 16%
Hyperthyroidism	- 18%	- 17%
Hypercortisolism	0%	0%
Compound 48/80 treated	0%	0%

ratio remained relatively higher, suggesting a contrasting effect of thyroid hormone on cardiac muscle compared with skeletal muscle (Fig. 4.1). These observations are similar to those reported by Carter *et al* (1980), who additionally measured protein breakdown by means of pulse labelling studies in myofibrillar and sarcoplasmic fractions of skeletal and cardiac muscles of hyperthyroid rats. They found that the skeletal muscle fractions had increased breakdown rates, while cardiac muscle fractions had decreased or normal rates.

4.3.2. *In vitro* enzyme activities

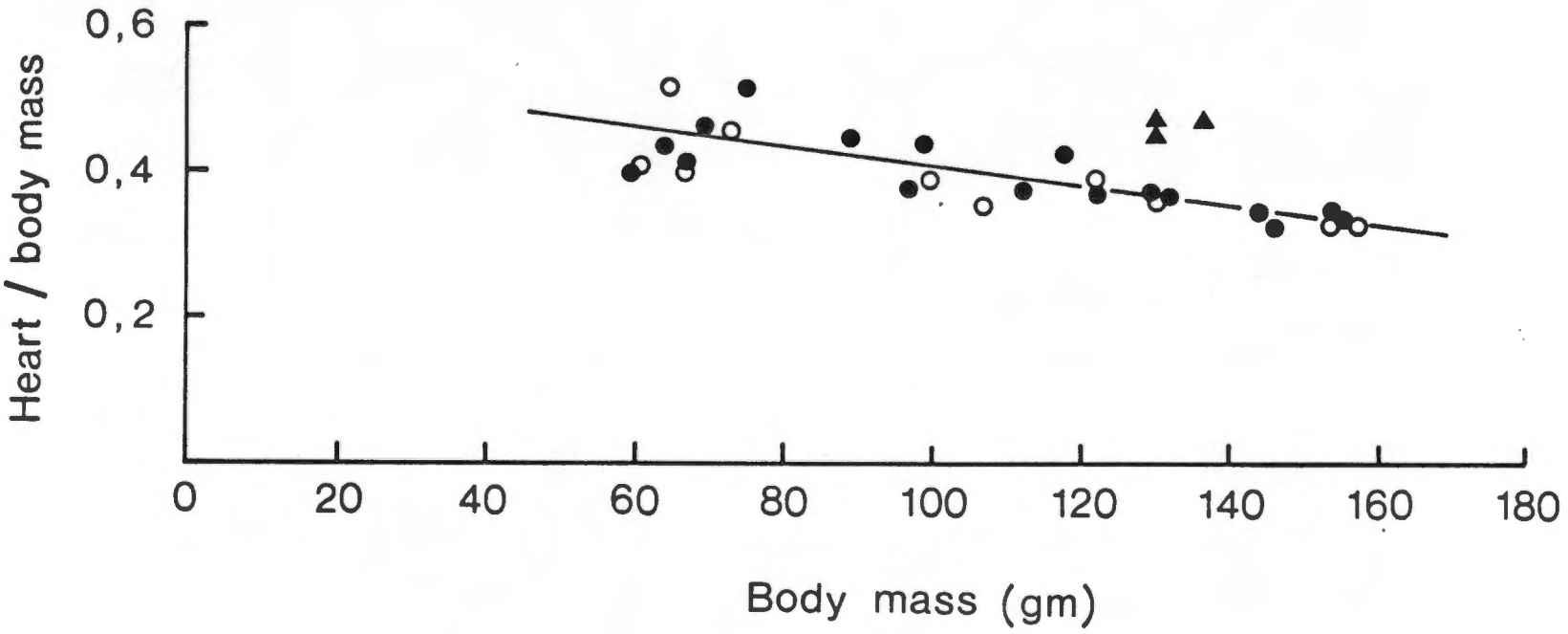
Compared with control rats, mast cell degranulation resulted in a marked decrease (> 90%) in chymase activity (Table 4.2), while the high molecular weight protease activity remained unchanged (Table 4.3). Not only was this confirmatory evidence for the mast cell location of chymase, but these observations also indicated that the high molecular weight protease was of myocytic origin.

Measurement of chymase and high molecular weight protease activities in the various myopathic groups (diabetes mellitus, hyperthyroidism, denervation and starvation) revealed no difference in these activities from those observed in muscles derived from control rats (Tables 4.2 and 4.3).

Although Mayer and Shafrir (1974) noted that starvation caused an increase in 'myofibril associated alkaline protease' activities in muscles, this increase was noted only after

Fig. 4.1

HEART/BODY MASS RATIOS VS BODY MASSES



The ratios of wet masses of hearts to body masses were compared with changing body masses in normal (o), manipulated (●) - (diabetic, starved, 48/80-treated, hypercortisol-treated) and hyperthyroid rats (▲).

4 days; in contrast, we noted that 2 days of starvation was already associated with increased *in situ* proteolysis, but not with changed chymase activity.

In the case of the high molecular weight protease, it was observed during its characterization (see Chapter 3) that the column treated HSSN fraction revealed a linear relationship with increasing protein concentration, whereas the activity in the relatively unpurified HSSN did not behave in this (desirable) way. For this reason, in this comparative study, the Sepharose 6B-treated material was pooled and compared, looking both at total and specific activities in the various groups tested. Both these parameters of activity did not change in parallel with enhanced *in situ* rates of proteolysis, implying that if one were to postulate a role for the high molecular weight protease in the pathway of protein degradation, it would have to assume one that was not rate-limiting for the overall degradative process (unless the concentrations of some critical and specific inhibitors were changing in these various states).

4.4. DISCUSSION

Rates of *in situ* proteolysis of intact soleus and EDL muscles were the same in controls and in rats subjected to mast cell degranulation. In addition, the extent of inhibition of proteolysis by known protease inhibitors (chymostatin and leupeptin) was of the same order in these two groups, while chymase activity had fallen markedly in the treated animals -

confirming earlier reports (Libby and Goldberg, 1980). These observations suggested that the overall degradative process (from intact proteins to the level of amino aicds) was not limited by the activity of the mast cell protease. Furthermore, we have observed that denervation in rats in whom the mast cells have been degranulated showed a similar enhancement of proteolysis as that observed in normal rats undergoing denervation - showing that the mast cell protease is not a limiting factor in altered states of proteolysis.

The activity levels of chymase were similar in all groups studied (with the exception of the mast cell degranulated group), revealing the lack of correlation between chymase activity and altered proteolytic states - a feature of a non-participant or a non-regulatory protease.

Similar activity levels of the high molecular weight protease were detected in muscles derived from rats subjected to mast cell degranulation and controls, suggesting a myocytic origin of this enzyme. The lack of correlation between altered *in situ* rates and the activity of this protease suggest that this protease is not rate-limiting for proteolytic processes. However, similarities between this protease and that of the reticulocyte system (Hershko *et al*, 1980) not only suggest that this enzyme may form part of a similar multi-component ATP-dependent system in muscle, but furthermore indicate that the regulatory factor(s) in protein degradation may only become more apparent as greater insight is gained into the components participating in the pathway

of protein degradation. The characterization of a mechanistic pathway in muscle protein degradation on the lines of the reticulocyte system (Hershko et al, 1980), holds the promise of answering such questions in the field of muscle biochemistry.

CHAPTER 5

CONCLUDING SUMMARY AND REMARKS

5. CONCLUDING SUMMARY AND REMARKS

Rat skeletal muscle homogenates contained an easily sedimented, chymostatin-sensitive protease(s) which acted on associated myofibrillar proteins at an optimum pH of 8.5. This action was less rapid at low than at high ionic strength, when myosin heavy chains were cleaved with the formation of heavy and light meromyosin-like fragments. The protease also acted on protein substrates present in the particle-free cytosol fraction of the muscle extracts, but this activity was more pronounced at low ionic strength (filamentous form of the myofibrillar proteins).

Inhibitor(s) of the proteinase were apparently present in the cytosol fraction; these did not act on the filamentous form of the enzyme, but were effective at high ionic strength when all the myofibrillar (and associated) proteins were in solution.

The enzyme was separated from the bulk of the myofibrillar proteins by gel chromatography at high ionic strength. On dialysis against a buffer at low ionic strength, part of the enzyme was precipitated. The putative cytosolic inhibitors were again only effective on the soluble enzyme at high ionic strength.

The proteinase activity virtually disappeared from the muscles of animals treated with the mast cell degranulator, compound 48/80. Furthermore, the rates of proteolysis in intact

soleus and extensor digitorum (EDL) muscles obtained from the same treated rats were not different from those found in muscles of matched controls. Extracts from a line of cultured skeletal muscle cells did not show such an enzyme activity in the particulate myofibrillar fraction.

Starvation, denervation and the induction of hyperthyroidism and diabetes mellitus in the laboratory rats, yielded muscles that displayed increased protein degradation, but these states were not associated with changes in the activity level of the myofibril-associated protease. This enzyme thus appeared to be the mast cell "chymase", released during homogenization of the muscle tissue, and was not of myocytic origin. However, its properties when associated with the myofibrils and when in free solution, present a model for the differential control of cellular proteinases by inhibitors whose effectiveness can be modulated by variable supramolecular structure.

Although the role of the mast cell in health and disease remains poorly understood (Pepys and Edwards, 1979), the data presented here indicate that the mast cell chymase is an unlikely participatant in proteolysis within muscle cells. Nevertheless, recent reports illustrating the possible synergism between chymase and calcium activated protease *in vitro* (Kay *et al*, 1981), suggest that chymase may still be a possible candidate for intracellular proteolysis and therefore further knowledge into precise biochemical events in the pathway of protein degradation remains to be obtained in order to credit this protease with its relevant function(s).

A cytosolic enzyme which attacks native or denatured proteins (casein, globin, hexokinase, mixed myofibrillar proteins) was detected and purified about 5000-fold from various rat skeletal muscles, including muscles freed of mast cells by prior treatment of the animals with the degranulator, compound 48/80. Peptides of varying size were generated from radio-actively labelled globin, but no free amino acids were formed; free tyrosine was also not released from azocasein. The pH optimum was 7,5 and the presence of an essential thiol group was suggested because dithiothreitol stimulated the activity and N-ethylmaleimide and parachloromercuriphenylsulphonic acid were inhibitors. The activity was markedly inhibited by Zn^{++} but not by leupeptin, chymostatin or pepstatin. The enzyme was stabilized by ATP, at concentrations as low as 0,1 mM, against inactivation at $42^{o}C$. The apparent molecular mass estimated by gel filtration was 500000 and SDS-polyacrylamide gel electrophoretic analysis of the partially purified enzyme showed that two subunits with approximate molecular weights of 100000 and 52000 represented most of the protein.

The endoprotease was clearly separated on gel chromatography from another large protease, also sensitive to Zn^{++}, but with marked aminopeptidase activity and the properties of Hydrolase H (Okitani et al, 1981).

No evidence for a ubiquitin cofactor in skeletal muscle was found; partially purified erythrocyte ubiquitin also did not produce ATP-dependence of the activity of the high molecular mass protease, at any level of purification.

The activity levels of the protease, assayed after chromatography on Sepharose 6B of high-speed supernatant fractions, did not vary significantly in skeletal muscle samples which were derived from denervated, starved, diabetic or hyperthyroid animals, in all of which the abnormal physiological states expressed themselves as enhanced rates of tyrosine release by incubated soleus and EDL muscles. Nevertheless, the enzyme described here may be part of an ATP-dependent, multi-component proteolytic system, similar to that already known to be present in reticulocytes.

The results presented here, suggest that this macroprotease is of myocytic origin and the lack of correlation of this activity with altered rates of proteolysis further indicate that this enzyme is not rate-limiting for the overall process of intracellular protein degradation. However, in terms of present pathway models, the role of this high molecular weight protease in intracellular protein degradation would be that of an extralysosomal non-regulatory protease.

The observation that physiological manipulation *in vivo* influences measured rates of proteolysis *in vitro* confirms previous reports, and in this regard an interesting hormonal effect is that of thyroid hormone. Thyroid hormones at physiological levels are necessary for growth, while the same in excess result in muscle wasting (Goldberg, 1980). With regard to the latter, while skeletal muscles atrophy and display enhanced proteolytic rates, cardiac muscle undergoes hypertrophy (Carter *et al*, 1980) - an observation which has

been confirmed in this work. A change in cardiac myosin heavy chain isozyme distribution in response to thyroid hormone (Hoh et al, 1977; Sartore et al, 1981) represents a model whereby thyroid hormones can initiate a switch in protein synthesis at a nuclear level accounting for the clinical effects of the hyperthyroid state. Further studies on a similar design examining skeletal muscles of hyperthyroid animals may well add clarification towards the contrasting effects of excess thyroid hormone on cardiac and skeletal muscles. The specific rate-limiting biochemical event(s) that are influenced by the endocrine system remains one challenging area in protein turnover research that holds the promise for the identification of appropriate therapy of muscle wasting disorders.

CHAPTER 6

TAKING STOCK

The skeletal muscle cell is composed of a number of sub-cellular compartments in which proteins in both particulate and soluble forms undergo differential rates of turnover. Several explanations for the overall function of continuous intracellular protein degradation have been proposed (Goldberg and Dice, 1974; Goldberg and St John, 1976; Ballard, 1977), but their significance remains uncertain. A central suggestion appears to be that turnover may facilitate the adaptation of the organism to its environment. In addition, adaptation to poor environments may require protein degradation simply to provide energy and sufficient amino acid precursors for the synthesis of those proteins which are required (such as catabolic enzymes). Another important function of protein degradation may be the removal of abnormal (defective) proteins produced as a result of exogenous (mutagens) or endogenous (biosynthetic error) factors.

There have been several difficult key questions posed to investigators in the study of skeletal muscle protein degradation. One of these is the significance of the differential turnover rates of the numerous cellular proteins in their native state.

A second major unresolved puzzle is how the "ageing" of a protein renders it "abnormal" and thus marked as a susceptible substrate for the intracellular degradative machinery. The energy-dependent multi-component system in reticulocytes as described by Hershko and co-workers (Hershko *et al*, 1979; Ciechanover, *et al*, 1980[b]) is one in which the denatured state

of substrate protein is a pre-requirement for this mechanism to be operative. The chemistry of this "ageing abnormality" may be mediated by processes such as methylation, phosphorylation or de-amidation. Support for such chemical modification resulting in an enhanced susceptibility of proteins to protease digestion has already been described by several independent investigators (Bohley *et al*, 1981; Pace *et al*, 1981, and Holzer, 1981). Against this "ageing" model is the "first order" model of the fate of intracellular proteins in which the random fate of all molecules (young or old) is the same. The two models need not necessarily be in opposition to each other as the "ageing" phenomenon could be a first order process, i.e. any protein molecule could age at any moment.

The third question awaiting an answer is how many differing pathways are present (viz. *lysosomal*, with or without autophagy, and *non-lysosomal*, energy-dependent and energy-independent) and how these may be inter-related in cellular proteolysis.

The fourth problem concerns the site of flux-generating step(s). The answer to this would make possible important advances in terms of knowledge of the physiology of protein degradation as well as specific therapy for muscle-diseased states. The altered rates of in-situ muscle proteolysis in response to changes in the hormonal status of experimental animals suggests that the well-known endocrine myopathies in human patients are

mediated by hormones acting at flux-generating reaction steps along the pathway. Hormones could result in regulation of absolute levels of proteases by activation-inactivation steps or by altering levels of endogenous inhibitors. Inhibitor effect could be modulated by changes in absolute inhibitor concentration, activation-inactivation chemical interconversions, or possibly even an extra-cellular source of specific protease inhibitors. Mammalian serum which contains several anti-proteases (Laskowski and Kato, 1980) is a potential source of extracellular regulators of muscle proteolysis in muscle injury or inflammation and other myopathic states. A number of investigators have demonstrated elevated activity levels of several proteases in muscle extracts from humans afflicted with muscular dystrophies (for review, see Kar and Pearson, 1980). However, as some of these proteases were muscle-cell derived while others were of non-muscle cell origin, the significance of these observations in terms of the regulation of proteolysis in muscle disease is unknown at present (Dahlmann et al, 1981b).

Finally, in addition to understanding the biochemical details of the homeostatic mechanisms within muscle fibres which serve to maintain a steady state of protein catabolism, the process by which protein synthesis is coordinated with degradation to maintain protein equilibrium is yet another area which awaits clarification.

These are all difficult questions and further experimental

work will be required to incorporate the present knowledge with the new in order to arrive at a composite mechanistic picture of the intracellular degradative system.

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